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(54) Title: TOPOLOGICALLY SEGREGATED, ENCODED SOLID PHASE LIBRARIES

(57) Abstract

The invention relates to libraries of synthetic test compound attached to separate phase synthesis supports. In particular, the invention relates to libraries of synthetic test compound attached to separate phase synthesis supports that also contain coding molecules that encode the structure of the synthetic test compound. The molecules may be polymers or multiple nonpolymeric molecules. Each of the solid phase synthesis support beads contains a single type of synthetic test compound. The synthetic test compound can have backbone structures with linkages such as amide, urea, carbamate (i.e., urethane), ester, amino, sulfide, disulfide, or carbon-carbon, such as alkane and alkene, or any combination thereof. Examples of subunits suited for the different linkage chemistries are provided. The synthetic test compound can also be molecular scaffolds, such as derivatives of monocyclic of bicyclic carbohydrates, steroids, sugars, heterocyclic structures, polyaromatic structures, or other structures capable of acting as a scaffolding. Examples of suitable molecular scaffolds are provided. The invention also relates to methods of synthesizing such libraries and the use of such libraries to identify and characterize molecules of interest from among the library of synthetic test compound.

TOPOLOGICALLY SEGREGATED, ENCODED
SOLID PHASE LIBRARIES

The present application is a continuation-in-part
5 of copending application Serial No. 08/068,327, filed
on May 27, 1993, which is incorporated herein by
reference in its entirety.

1. FIELD OF THE INVENTION

10   The invention relates to libraries of synthetic
test compound attached to separate phase synthesis
supports. In particular, the invention relates to
libraries of synthetic test compound attached to
separate phase synthesis supports that also contain
15 coding polymeric sequences that encode the structure
of the synthetic test compound. Each of the solid
phase synthesis support beads contains a single type
of synthetic test compound and a single, unique and
readily determinable coding polymeric sequence
20 encoding the structure of the synthetic test compound.
The synthetic test compound can have backbone
structures with linkages such as amide, urea,
carbamate (i.e., urethane), ester, amino, sulfide,
disulfide, or carbon-carbon, such as alkane and
25 alkene, or any combination thereof. The synthetic
test compound can also be molecular scaffolds, such as
derivatives of monocyclic or bicyclic carbohydrates,
steroids, sugars, heterocyclic structures,
polyaromatic structures, or other structures capable
30 of acting as a scaffolding. The invention also
relates to methods of synthesizing such libraries and
the use of such libraries to identify and characterize
molecules of interest from among the library of
synthetic test compound.

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2.   <u>BACKGROUND OF THE INVENTION</u>

Ligand recognition and binding regulates almost
all biological processes, including immune
recognition, cell signalling and communication,
5   transcription and translation, intracellular
signalling, and enzymatic catalysis. As a result,
there is a longstanding interest in the art in
identifying molecules which can be used as follows:
to serve as agonists or antagonists of ligands such as
10   hormones, growth factors, or neurotransmitters; to
induce B-cell (antibody-mediated) or T-cell (cell-
mediated) immunity; to induce catalysis of chemical
reactions; and to regulate gene expression at the
level of transcription or translation. A main reason
15   for this interest is the desire to directly use these
biologically active molecules as drugs or, if
necessary, to convert these molecules into derivatives
which can function as drugs.

Many biological ligands are proteins or peptides.
20   This list includes the majority of hormones, growth
factors, neuroactive molecules, and immune epitopes.
For this reason, initial efforts to develop agonists
or antagonists of receptor- or enzyme-mediated
biological activities involved peptide design and
25   synthesis. However, peptides that have been found to
possess desirable biological activities are often
unsuitable as drugs. To become drugs, the peptides
often need to be converted to derivatives or
structural analogs, i.e., peptide mimetics, which,
30   unlike most peptides, possess satisfactory
pharmacokinetics and stability properties. Many
publications describing the development of medicinally
useful or promising peptidomimetics have appeared;
some recent examples include Rudy Baum, in *Chemical &*
35   *Engineering News, January 18, 1993, page 33;*

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Hirschmann, R. et al. *J. Am. Chem. Soc.*, *1992*, *114*,
9699-9701; Hirschmann, R. et al. *J. Am. Chem. Soc.*,
1992, *114*, 9217-9218.

The discovery of biologically active compounds

5   can be a difficult, time-consuming, and extremely
expensive process. A key problem in this area is the
identification of a single chemical structure, out of
a large number of possible relevant structures, that
possesses the desired properties. When the discovery

10 process employs a sequential strategy of
structure-design, synthesis, and biological testing,
the identification of a desirable chemical structure
becomes extremely laborious. To circumvent this
highly demanding task, libraries of large numbers of

15 molecules of diverse structures can be prepared.
Ideally such libraries can be screened and evaluated
rapidly.

Much of the work in this area of library
synthesis and screening has been done with peptides,

20 e.g., the approaches of Geysen (Geysen et al.
Molecular Immunology, *1986*, *23*, 709-715; Geysen et al.
J. Immunologic Methods, *1987*, *102*, 259-274), Fodor
(Fodor et al., *Science*, *1991, 251*, 767-773) and
Houghten (Houghten et al., *Nature*, *1991, 354*, 84-86).

25 However, such libraries are limited in terms of the
number of possible structural variants that can be
prepared, tested and identified in a given experiment.

The invention of truly random libraries of
polymeric synthetic test compound, in which a single

30 polymeric species arising from a combination of
subunits is attached to a single solid support, marked
a breakthrough in the discovery of biologically active
compounds which are peptides or, very importantly,
peptide mimetics (see, U.S. patent application Serial

35 No. 07/717,454, filed June 19, 1991, entitled "Random

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Bio-Oligomer Library, a Method of Synthesis Thereof, and a Method of Use Thereof" and U.S. patent application Serial No. 07/456,845, filed July 2, 1990, entitled "Random Peptide Library, a Method of

5   Synthesis Thereof and a Method of Use Thereof").

Nonpeptidic organic compounds, such as peptide mimetics, can often surpass peptide ligands in affinity for a certain receptor or enzyme. The binding of biotin and avidin, the tightest ever

10 recorded, involves association of a non-peptidic organic structure (biotin) with a protein (avidin). An effective strategy for rapidly identifying high affinity biological ligands, and ultimately new and important drugs, requires rapid construction and

15 screening of diverse libraries of non-peptidic structures containing a variety of structural units capable of establishing one or more types of interactions with a biological acceptor (e.g., a receptor or enzyme), such as hydrogen bonds, salt

20 bridges, π-complexation, hydrophobic effects, etc. However, work on the generation and screening of synthetic test compound libraries containing nonpeptidic molecules is now in its infancy. One example from this area is the work of Ellman and Bunin

25 on a combinatorial synthesis of benzodiazepines on a solid support (*J. Am. Chem. Soc. 114, 10997, (1992); see Chemical and Engineering News, January 18, 1993, page 33*).

A key unsolved problem in the area of generation

30 and use of nonpeptide libraries is the elucidation of the structure of molecules selected from a library that show promising biological activity.

An attempt to uncover the structures of peptides selected from a library using unique nucleotide

35 sequence codes, which are synthesized in tandem with

the peptide library, has recently been described by
Brenner and Lerner (Brenner, S. and Lerner, R.A. *Proc.
Nat'l. Acad. Sci. USA, 1992 89*, 5381-5383). The
nucleotide sequence of the code attached to each
5 peptide must be amplifiable via the polymerase chain
reaction (PCR). However, nucleotide synthesis
techniques are not compatible with all of the
synthetic techniques required for synthesis of many
types of molecular libraries. Furthermore, the close
10 proximity of nucleotide and synthetic test compound in
the library, which can result in interactions between
these molecules interfering with the binding of the
ligand with a target receptor or enzyme during the
biological assay, also limits this approach. The
15 nucleotide component of the library can also interfere
during biological assays in a variety of other ways.

Kerr et al. (*J. Am. Chem. Soc., 1993, 115*, 2520-
2531) reported synthesizing solution phase libraries
of peptides, containing non-natural amino acid
20 residues, in parallel with peptide coding strands.
The peptide ligand and its coding strand in this
library are covalently joined together, which allows
isolation and sequence determination of pairs of
synthetic test compound and corresponding code.
25 However, as with the nucleic-acid-encoded library
described by Brenner and Lerner, *supra*, the coding
peptide may interfere with the screening assay.
Moreover, the requirement for purification of
sufficient amounts of material from the library with
30 the affinity selection method, in order to obtain the
sequence of the coding peptides, precludes synthesis
of libraries of more than a few thousand species.

Thus, there is a need in the art for new,
general, and versatile methods for generating and
35 screening libraries of compounds belonging to a

variety of chemical classes. There is a further need
for effective methods for elucidating the structures
of compounds selected from the library as a result of
screening, whose structures cannot be determined by

5 traditional techniques, e.g., Edman degradation or
mass spectrometry alone. Yet another need in the art
is a molecular coding system that will not interact in
screening assays or influence the binding of the
synthetic test compound through proximity effects.

10   Citation or identification of any reference
herein shall not be construed as an admission that
such reference is available as prior art to the
present invention.

15   3. **SUMMARY OF THE INVENTION**
The present invention is directed to libraries of
synthetic test compound attached to separate phase
supports, in which a single species of compound is
attached to each support. The compounds can have

20 linkages such as, but not limited to, amide, urea,
ester, ether, carbamate, amine, sulfide, disulfide,
carbon-carbon, such as alkane, alkene and alkyne, and
the like. In particular, the compounds can be
polyamides, polyureas, polyurethanes, polyesters,

25 polyethers, polycarbonates, polyamines, polyalkanes,
polysulfides, polydisulfides, or polymers containing
any combination of such bonds. The compound may also
be a molecular scaffold having various substituents at
defined positions, in which the scaffold can be a

30 cyclic or bicyclic hydrocarbon, a steroid, a sugar, a
heterocyclic structure, or a polycyclic aromatic
molecule.
The present invention further relates to encoded
libraries of synthetic test compound, in which a

35 sequence of a coding molecule on a separate phase

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support corresponds exactly to, *i.e.*, encodes, the
synthetic test compound attached to the support. In a
preferred embodiment, the coding molecule is a
peptide. An alternative embodiment encompasses the

5   coding of a library of sequencable test compounds,
wherein certain subunits are indistinguishable by
conventional sequencing techniques. The coding is
accomplished by adding a small fraction of a third
distinguishable coding subunit to the coupling

10   mixture. In this coding technique, called fractional
coding, there is no discrete coding molecule at all.

The invention also provides encoded libraries in
which the synthetic test compound is topologically
segregated from the coding molecule on each separate

15   phase support. In a preferred embodiment, in which
the separate phase support is a resin bead, the
synthetic test compound is located on the surface of
the resin bead while the coding molecule is located in
great abundance in the interior of the bead. In

20   another embodiment, the synthetic test compound is
attached to the separate phase support by a cleavable
linker, and the coding molecule is attached to the
support with a non-cleavable linker or a separately
cleavable linker. In this embodiment, during a

25   screening assay, the synthetic test compound can be
released from the separate phase support, *e.g.*, into
solution, while the coding molecule remains attached
to the support.

The present invention is also directed to methods

30   of synthesis of the libraries of synthetic test
compound and encoded libraries of synthetic test
compound. Synthesis of the synthetic test compound
involves separation of portions of the separate phase
supports, and reaction with a single subunit of the

35   synthetic test compound with each portion under

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conditions such that the added subunit can react with
functional groups on the synthetic test compound. If
an encoded library is to be prepared, the subunit of
the coding molecule that corresponds to the subunit of
5   the compound is added to each of the separated
portions before they are recombined, using a separate
set of reaction conditions such that the coding
subunit will only react with the coding components of
the separate phase support. The order of reaction,
10   described as linking the synthetic test compound
subunit followed by the coding subunit is not
material. The order could just as well be coding
subunit reaction followed by the synthetic test
compound subunit. What is important is that both
15   reactions are carried out on the same portion of the
solid phase support.
        After the separate coupling steps, the portions
are thoroughly mixed in order to randomize them. The
process of separate coupling and thorough mixing is
20   repeated until as many subunits of the compound have
been added as is desired. Then any functional group
protecting groups that remain on the compounds (and
the coding sequence, if any) are removed, without
cleaving either the compounds or the coding molecules
25   from the separate phase support.
        The present invention is also directed to methods
for screening the libraries. The libraries can be
screened for binding activity of an acceptor molecule
on the separate phase support, or for binding activity
30   of the released compound. The invention further
provides for biological screening assays of the
released compounds.
        Other screening assays for the compounds, such
as, but not limited to, enzyme activity, electron
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transport activity, and photo activity, to mention a few, are also contemplated by the invention.

Solid supports that contain compounds that demonstrate the activity of interest in the screening
5   assay are selected. The structure of the compound is determined, e.g., by mass spectrometry, nuclear magnetic resonance spectrometry, or other spectrometric methods. Preferably, the library is an encoded library, in which case the structure of the
10 compound is encoded by the sequence of the coding molecule, which can be readily determined.

The compounds of the present invention can provide leads for therapeutic or diagnostic agents. More preferably, the compounds themselves may be
15 useful therapeutic or diagnostic agents. The compounds on separate phase supports can also be useful for electron transport, e.g., as transistors or semiconductors.

20   4. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Strategies for attaching encoded libraries of synthetic test compound. Subunits of the synthetic test compound are indicated as [NONSEQ]; the coding molecule is indicated by A-B-C. (A) The test
25 compound and coding molecule can be attached separately to the support directly or via a linker, in a statistical distribution that can be altered based on mass action and inherent reactivity. (B) The synthetic test compound and the coding molecule can be
30 attached to the same linker on the separate phase support, in a defined molar ratio. (C) The synthetic test compound is attached on the surface and the coding molecule is attached in the interior of a separate phase support, such as a resin bead.
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Alternatively, in (C), the coding molecule can be on the surface and the test compound in the interior.

Figure 2. Three forms of a model encoded library in which the "test" compound (Ala-Phe-Val) and the
5 coding molecule (Gly-Tyr-Leu) are both peptides. (A) The "test" compound was synthesized using the Fmoc protecting group, and the coding molecule was sythesized with the Boc protecting group. Fmoc and Boc are orthogonal protecting groups. (B) The Fmoc
10 deprotected "test" compound was acetylated so that only the coding peptide would be sequenced by Edman degradation. (C) The coding peptide was blocked with trifluoroacetyl (TFA), allowing Edman sequencing of the "test" compound, followed by removal of TFA and
15 sequencing of the coding peptide. The peptides were attached to the TentaGel (TG) resin via a safety-catch amide linker (SCAL; Patek and Lebl, 1991, Tetrahedron Lett. 32:3891-3894) with lysine branches.

Figure 3. Model branched scaffold libraries,
20 showing various possible linking chemistries, including reaction of an amine with a carboxylic acid to form an amide; reaction of a carboxylic acid with an amine to form an amide; reaction of a thiol with a alkylhalogenid to form a sulfide.

25 Figure 4. A model of interaction of a scaffold synthetic test compound with an acceptor molecule. (A) The functional groups attached to the scaffold are free to assume an appropriate binding conformation. (B) The functional groups on the
30 scaffold are constrained in the appropriate binding conformation.

Figure 5. Structures of some of the subunits that can be chemically linked in random structures to form the libraries of synthetic test compound.

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Figure 6. A model cyclic library formed from repeated condensation reactions with Boc and Fmoc blocked subunits.

Figure 7. A scheme for preparation of a scaffold
5   library on TentaGel resin in which the scaffold is cyclopentane.

Figure 8. Structures of subunits used to prepare a scaffold library of the invention. The two-letter amino acid dipeptide code for each of the subunits is
10 shown below each one. Preparation and use of this library is described in Section 9, *infra*.

5.   <u>DETAILED DESCRIPTION OF THE INVENTION</u>

The invention relates to libraries of synthetic
15 test compound attached to separate phase supports, in which each separate phase support contains a single species of synthetic test compound, and methods of synthesis and use of such libraries. The term "separate phase support" refers to a matrix to which
20 the synthetic test compound can be attached, which matrix is not soluble in a liquid or forms a two phase system with a liquid. Preferably, the separate phase is a solid phase, although separate phases such as hydrogels and aerogels are also contemplated.

25   As used herein, the term "library of synthetic test compound" refers to collections of synthetic test compound on separate phase support particles in which each separate phase support particle contains a single structural species of the synthetic test compound.
30 Each support contains many copies of the single structural species. For example, a typical resin support for solid phase peptide synthesis contains about 50-250 pmol of peptide. The structures of the synthetic test compound are derived from a

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substantially random chemical combination of "subunits".

As used herein, the term "synthetic test compound" refers to small molecules consisting of 2 to 5 100, and more preferably, 2-20, subunits, with or without a scaffold. In one embodiment, the synthetic test compound is a polymer formed of subunits linked via such linkages as amide, urea, ester, ether, carbamate, amine, sulfide, disulfide, carbon-carbon, 10 such as alkane, alkene and alkyne, and the like; in particular, the compound can include, but is not limited to, polycarbamate, polyurea, polyamide, polyester, polyether, etc., or any combination thereof, as described in detail *infra*. In another 15 embodiment, a synthetic test compound may be a randomly functionalized molecular scaffold, such as, but not limited to, a steroid, heterocyclic structure, a polyaromatic ring, or carbohydrate structure, and the like, as described in detail *infra*.

20   As used herein, the term "subunit" refers to a chemical subcomponent, whereby the synthetic test compound is formed by linkage of the chemical subcomponents by a defined chemistry. For example, a "library of peptides" is a collection of peptides (the 25 synthetic test compound), *i.e.*, chains consisting of 2-100 α-amino acid residues (the subunits), whose sequences contain any amino acid residue preceding or following any other amino acid residue. An example of a "library of steroid derivatives" is a collection of 30 steroid derivatives containing any one of a set of functional groups, the subunits, at specific positions of the steroid nucleus.

More preferably, the present invention relates to encoded libraries of synthetic test compound. As used 35 herein, the term "encoded library" refers to a library

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in which each distinct species of compound is paired on each separate phase support with a coding molecule whose structure is readily determinable and encodes a unique structure for its pair partner in the library.

5   In a preferred embodiment of an encoded molecular library, the coding polymeric molecule is a peptide. In another embodiment, the coding polymeric molecule is an oligonucleotide.

Examples of embodiments of libraries include, but
10   are not limited to, the following:

libraries in which the synthetic test compound are polyamides, i.e., the synthetic test compound are chains of 2-100 amino acids linked through amide bonds;

15   libraries in which the synthetic test compound are polyesters, i.e., chains of 2-100 hydroxy acids linked by ester bonds;

libraries in which the synthetic test compound are polyethers, i.e., chains of 2-100 hydroxy alcohols
20   linked by ether bonds;

libraries in which the synthetic test compound are polyureas;

libraries in which the synthetic test compound are polyurethanes;

25   libraries in which the synthetic test compound are polycarbonates;

libraries in which the synthetic test compound are polyamines;

libraries in which the synthetic test compound
30   are polyalkanes, polyalkenes, or polyalcohols, including halo derivatives thereof;

libraries in which the synthetic test compound are polysulfides;

libraries in which the synthetic test compound
35   are polydisulfides;

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libraries in which the synthetic test compound
are polymers whose structures contain randomly
arranged segments from two or more of the polymeric
structures described in the embodiments above;

5   libraries in which the synthetic test compound
are derivatives of a steroid structure;

libraries in which the synthetic test compound
are derivatives of a sugar such as β-D-glucose;

libraries in which the synthetic test compound
10   are derivatives of a heterocyclic structure, such as
benzodiazepine;

libraries in which the synthetic test compound
are derivatives of a structure capable of serving a
scaffolding onto which multiplicity of structures such
15   as but not limited to carboxylic acids, amines, and
halogen derivatives can be attached in a defined way;

libraries in which the molecules are chimeric
structures containing one or more sequences of
variable length linked by chemistry selected from one
20   or more of the following: amides, esters, ethers,
carbonates, sulfides, disulfides, alkenes, and amines,
and one or more structures capable of acting as a
scaffolding, such as a steroid, a sugar, an aromatic
or polyaromatic structure.

25   Many different subunits for the different classes
of synthetic test compound are commercially available
from suppliers such as Sigma, Aldrich, ICI Chemicals,
etc. Alternatively, subunits can be prepared
synthetically using standard chemical synthesis
30   techniques.

In a preferred embodiment, the libraries listed
above are encoded libraries in which each separate
phase support contains a synthetic test compound and a
polymeric sequence encoding the structure of the

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synthetic test compound. Preferably, the coding
polymeric sequence is a peptide.

5.1 CODING STRATEGIES

5 As noted above, in a preferred aspect the
libraries of the invention are encoded libraries in
which the sequence of a coding molecule on each
support corresponds to the structure of the synthetic
test compound on each support. Thus, each unique

10 synthetic test compound structure is encoded by a
unique coding molecule sequence. As noted *supra*,
preferably the coding molecule is a peptide, although
the present invention encompasses the use of nucleic
acids or any sequenceable polymer as a coding

15 sequence.
     The paradigm of a coding sequence is the genetic
code, in which triplet nucleotide sequences in a gene
corresponds to a specific amino acid in a protein
encoded by the gene. The arrangement of codons in a

20 gene corresponds to the sequence of amino acids in a
protein. Thus, the gene encodes the protein.
     Following this analogy, coding of the sequence of
a library of synthetic test compound, such as a
polyamide whose sequence cannot be established by

25 traditional methods (e.g., Edman degradation), with a
coding molecule can be accomplished readily using an
analogous code. The choice of the code is purely
arbitrary, including whether single, double or triple
(or greater) combinations of subunits of the coding

30 molecule correspond to each subunit of the synthetic
test compound.
     For example, the coding molecule may be a
peptide. In this case codes consisting of one or more
amino acid residues which can be readily detected by

35 Edman degradation, and are also known to couple

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efficiently in solid phase peptide synthesis without
requiring side-chain protection, are considered to be
especially useful. For example, if a triplet code
based on the amino acids leucine (Leu), glycine (Gly),
5   alanine (Ala), and phenylalanine (Phe) (all of which
do not require side chain protection and couple
efficiently during peptide synthesis and are
furthermore readily detectable by Edman degradation)
is used, libraries of synthetic test compound
10 containing up to sixty-four structurally different
subunits, with each subunit paired with a unique
peptide containing triplets of Leu, Gly, Ala, or Phe
(each triplet corresponding to one and only one
subunit in the polyamide), may be synthesized using
15 suitable chemical reactions. Other preferred amino
acids, i.e., those that do not require side chain
protection, include but are not limited to isoleucine,
valine, cyclohexyl-L-alanine, norleucine, norvaline,
proline, and the like. Less preferred are asparagine
20 and glutamine. In another embodiment, each of the 20
natural amino acids can code for a specific subunit.
A single coding sequence subunit or codon can code for
more than one subunit of the synthetic test compound,
resulting in a degenerate code, although this is not
25 necessary.
     The present invention provides various strategies
to increase the probability that screening assays
recognize active synthetic test compound instead of
the coding molecules on a given support, which are
30 discussed in Section 5.3., infra.

          5.2 METHODS FOR GENERATING A LIBRARY OF
               SYNTHETIC TEST COMPOUND

     As stated above, the present invention relates to
35 a method of generating a library of synthetic test

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compound on separate phase supports. Preferably the
library is one in which each synthetic test compound
is paired with a unique coding molecule, e.g., a
peptide, whose sequence encodes the structure of the
5   synthetic test compound attached to the same support
and can be readily determined using traditional
analytical techniques, e.g., Edman degradation.
        If the synthetic test compound are functionalized
molecular scaffolds, the scaffold or a precursor to
10 the scaffold will be attached to the solid phase
support prior to initiation of synthesis.
        The synthesis of libraries of synthetic test
compound comprises repeating the following steps:
        (i)   dividing the selected support into a
15 number of portions which is at least equal to the
number of different subunits to be linked;
        (ii) chemically linking one and only one of
the subunits of the synthetic test compound with
one and only one of the portions of the solid
20 support from step (i), preferably making certain
that the chemical link-forming reaction is driven
to completion to the fullest extent possible;
        (iii) thoroughly mixing the solid support
portions containing the growing synthetic test
25 compound;
        (iv) repeating steps (i) through (iii) a
number of times equal to the number of subunits
in each of the synthetic test compound of the
desired library, thus growing the synthetic test
30 compound;
        (v)   removing any protecting groups that
were used during the assembly of the synthetic
test compound on the solid support.
        Preferably, a coding molecule is synthesized in
35 parallel with the synthetic test compound. In this

instance, before or after linking the subunit of the synthetic test compound to the support in step (ii), one or more subunits of the coding molecule, that correspond(s) to the added subunit of the synthetic

5   test compound, is linked to the growing coding molecule such that a unique structural code (see Section 5.1, *supra*), corresponding to the structure of the growing synthetic test compound, is created on each support. It can be readily appreciated that if

10   an encoded library is prepared, synthesis of the coding subunit or subunits must precede the mixing step, (iii).

    The repetition of steps (i)-(iii) (see step (iv)) will naturally result in growing the synthetic test

15   compound and, if the process is modified to include synthesis of a coding molecule, the coding molecule in parallel with the test compound. The test arm and coding arm are used herein to refer to the synthetic test compound synthesized on the support, and, if

20   present, the coding molecule synthesized on the support, respectively.

    The present invention encompasses modification of any of the steps of the above procedure. For example, a different, and occasionally desirable, library will

25   result if step (ii) is changed to involve linking of the same polymer subunit to all of the portions of the solid support. In this case, elongation of the coding polymer needs to be modified analogously.

    In another embodiment, if the same polymer is

30   elongated on the test arm as on the coding arm, the coding arm need not be elongated past the point required to encode a non-sequencable subunit of the synthetic test compound, as long as the history of the synthesis is known, e.g., the number of subunits is

35   known.

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In another embodiment of the invention, the solid support used to carry out the synthesis of a synthetic test compound which is a short polymer is derivatized with one or more of the subunits of the polymer prior 5 to its use in the synthesis of a library.

In one embodiment, enough support particles are used so that there is a high probability that every possible structure of the synthetic test compound is present in the library. Such a library is referred to 10 as a "complete" library. To ensure a high probability of representation of every structure requires use of a number of supports in excess, e.g., by five-fold, twenty-fold, etc., according to statistics, such as Poisson statistics, of the number of possible species 15 of compounds. In another embodiment, especially where the number of possible structures exceeds the number of supports, not every possible structure is represented in the library. Such "incomplete" libraries are also very useful.

20   In a further embodiment, a library can have synthetic test compound whose structures include a desirable polymeric sequence, found as a result of screening another library added prior to generation of the library or at the end of generation of the 25 library. Such a library is prepared by synthesizing a solid support containing the desirable polymeric sequence and using this derivatized support as the solid support for the synthesis of the new library. Alternatively, a portion of the library synthetic test 30 compound can be synthesized followed by synthesis of the desirable polymer sequence as an extension of the test compounds on all of the separate phase supports. Alternatively, the desirable sequence may be discontinuous and included within the random library.

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5.3 DEVELOPMENT AND USE OF SEPARATE PHASE SYNTHESIS SUPPORTS AND LINKERS IN ENCODED MOLECULAR LIBRARY SYNTHESES

5.3.1 SUPPORTS AND LINKERS USEFUL IN ENCODED MOLECULAR LIBRARY SYNTHESIS

A separate phase support suitable for use in the present invention is characterized by the following properties: (1) insolubility in liquid phases used for synthesis or screening; (2) capable of mobility in three dimensions independent of all other supports; (3) containing many copies of each of the synthetic test compound and, if present, the coding sequence attached to the support; (4) compatibility with screening assay conditions; and (5) being inert to the reaction conditions for synthesis of a test compound and for coding molecule synthesis. A preferred support also has reactive functional groups, such as hydroxyl, carboxyl, amino, thiol, etc., for attaching a subunit which is a precursor to each of the synthetic test compound and coding molecules, or for attaching a linker which contains one or more reactive groups for the attachment of the monomer or other subunit precursor.

As used herein, separate phase support is not limited to a specific type of support. Rather a large number of supports are available and are known to one of ordinary skill in the art. In a preferred aspect, the separate phase support is a solid phase support, although the present invention encompasses the use of semi-solids, such as aerogels and hydrogels. Solid phase supports include silica gels, resins, derivatized plastic films, glass beads, cotton, plastic beads, alumina gels, polysaccharides such as Sepharose and the like, etc. A suitable solid phase support may be selected on the basis of desired end use and suitability for various synthetic protocols.

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 17/00; C12Q 1/68; C12P 21/00
US CL : 530/332, 323, 326, 327, 328; 435/6, 68.1

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   530/332, 323, 326, 327, 328; 435/6, 68.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Nature, Volume 354, Number 7, issued 07 November 1991, Lam et al., "A new type of synthetic peptide library for identifying ligand-binding activity", pages 82-84, see entire document.	1-54
A	Bioorganic & Medicinal Chemistry Letters, Volume 3, Number 3, issued 1993, "The chemical synthesis of large random peptide libraries and their use for the discovery of ligands for macromolecular acceptors", pages 419-424, see entire document.	1-54

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

Date of the actual completion of the international search	Date of mailing of the international search report
14 JULY 1994	AUG 11 1994

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Gary L. Brown
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

[NONSEQ]-[NONSEQ]-[NONSEQ]

Carrier

A-B-C

FIG. 1A

[NONSEQ]-[NONSEQ]-[NONSEQ]

Carrier

A-B-C

FIG. 1B

[NONSEQ]-[NONSEQ]-[NONSEQ]

A-B-C

FIG. 1C

Fmoc-(Ala-Phe-Val) ─────┐
                        │
Fmoc-(Ala-Phe-Val)─Lys ─┐
                        │
Boc-(Gly-Tyr-Leu)─Lys─SCAL─(TG)

FIG. 2A

Ac-(Ala-Phe-Val)─────┐
                     │
Ac-(Ala-Phe-Val)─Lys ┐
                     │
H(Gly-Tyr-Leu)─Lys─SCAL─(TG)

FIG. 2B

H-(Ala-Phe-Val)─────┐
                    │
H-(Ala-Phe-Val)─Lys ┐
                    │
TFA-(Gly-Tyr-Leu)─Lys─SCAL─(TG)
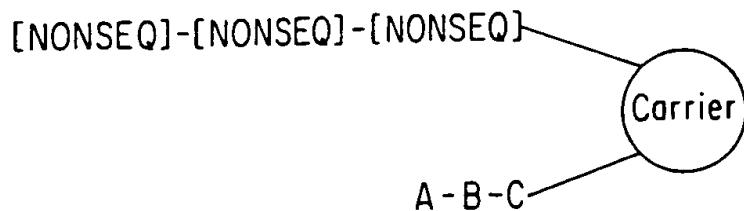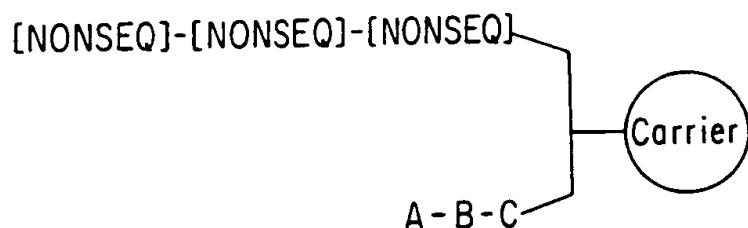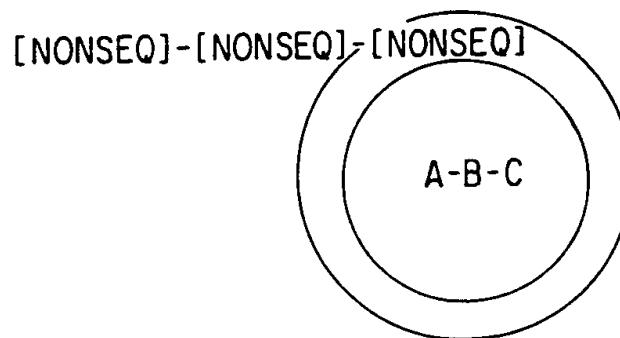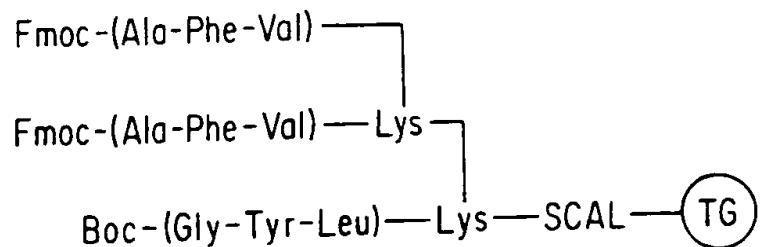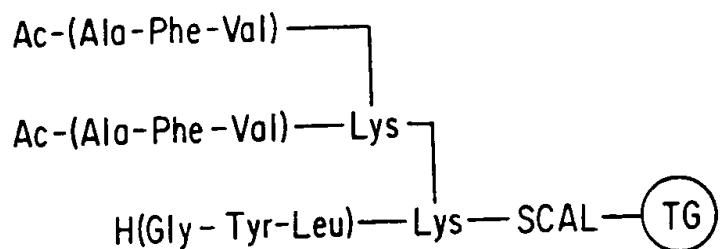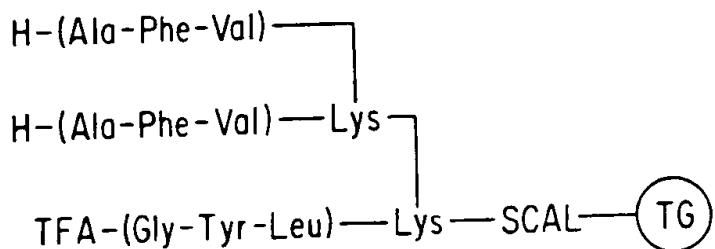
FIG. 2C
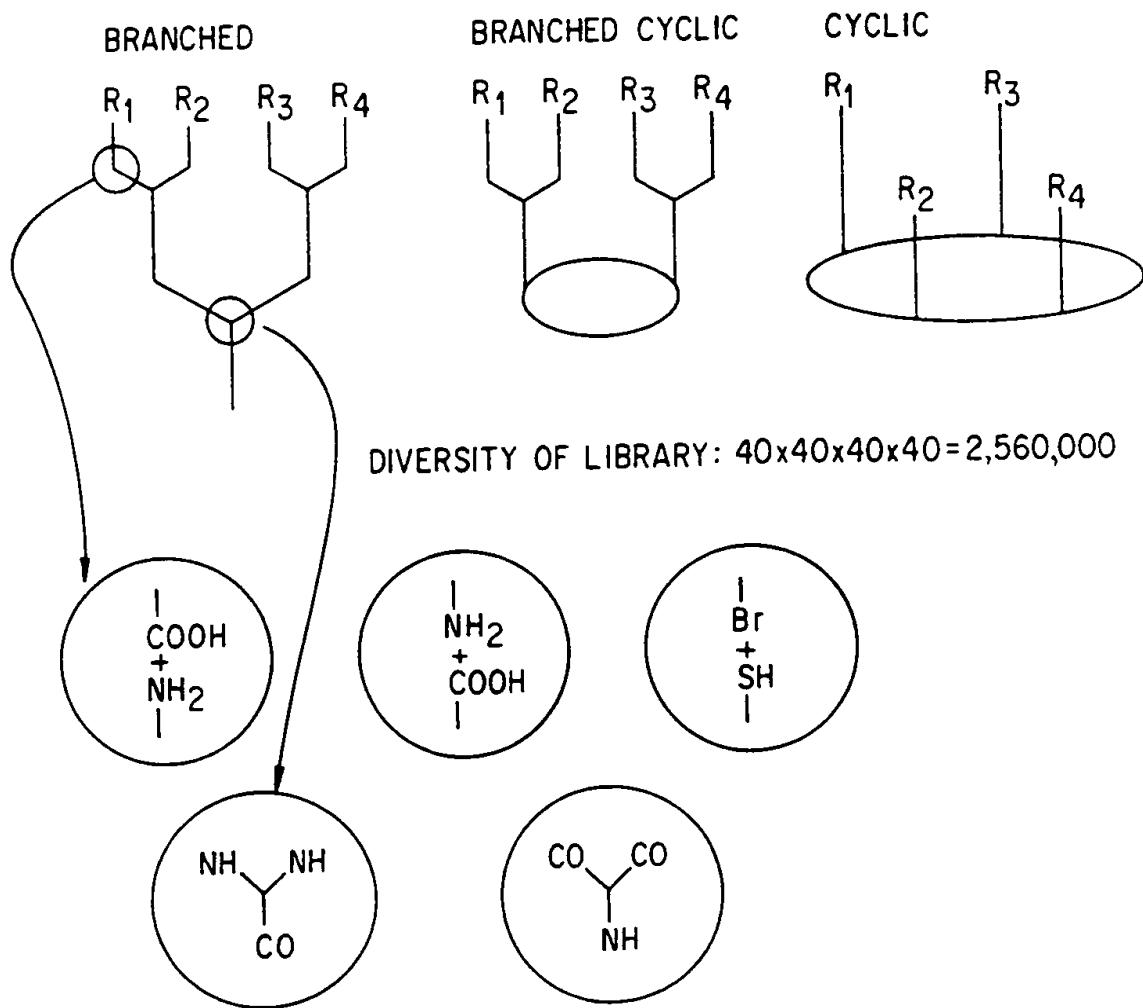
FIG. 3
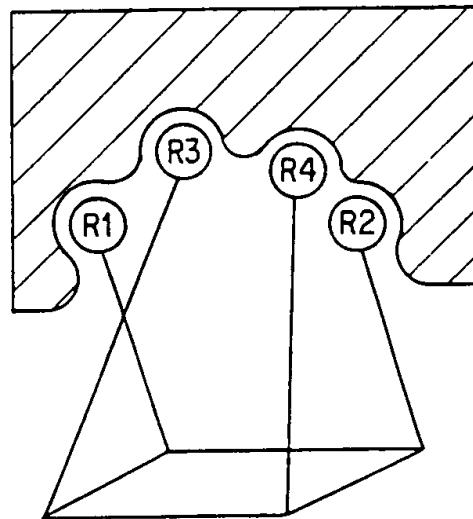
F I G. 4A
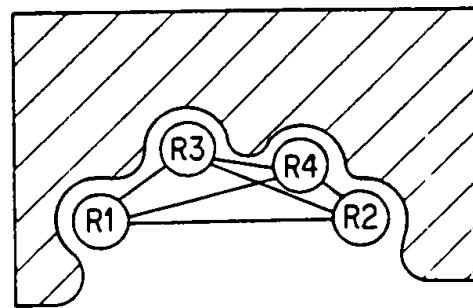
F I G. 4B
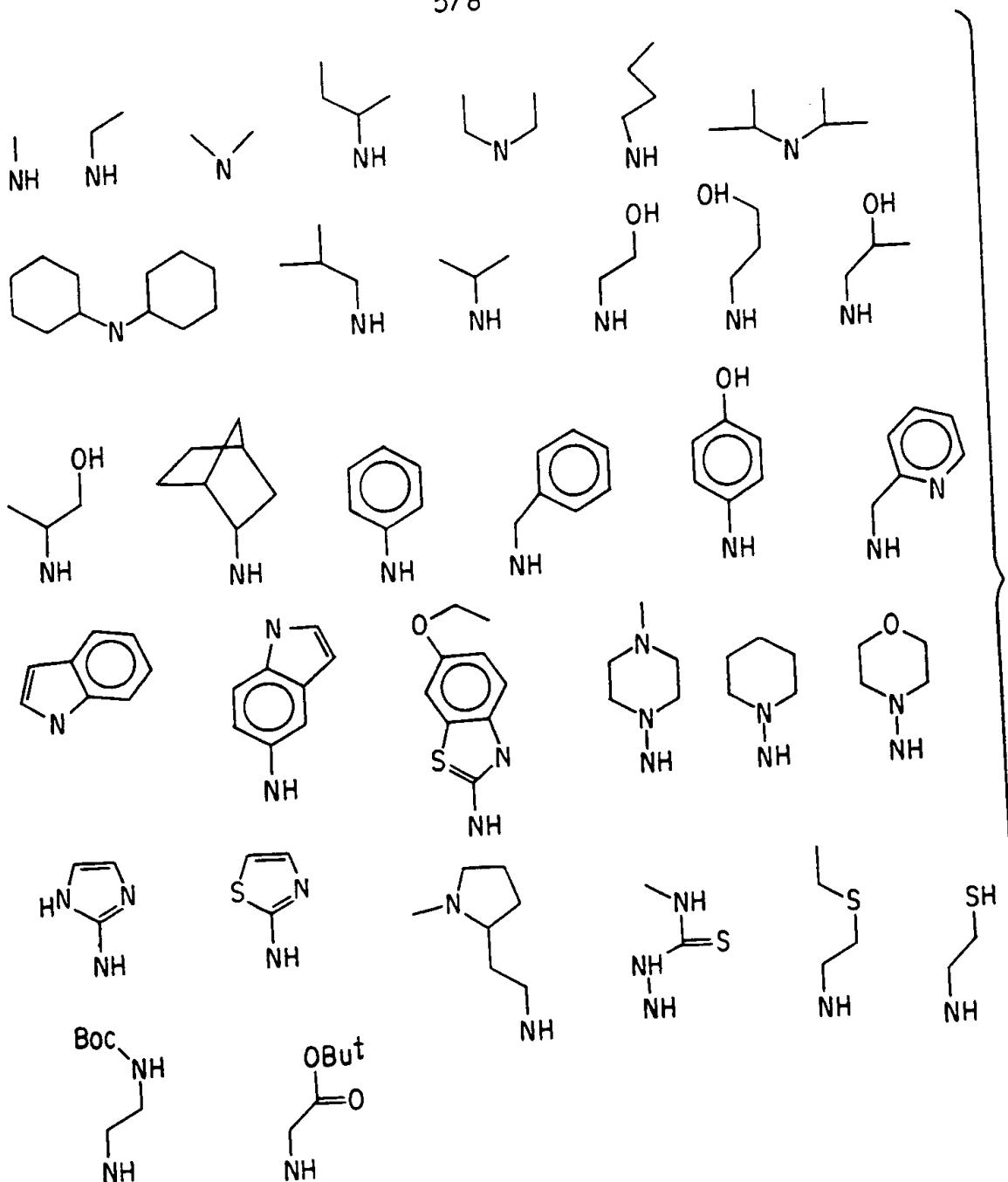
FIG. 5
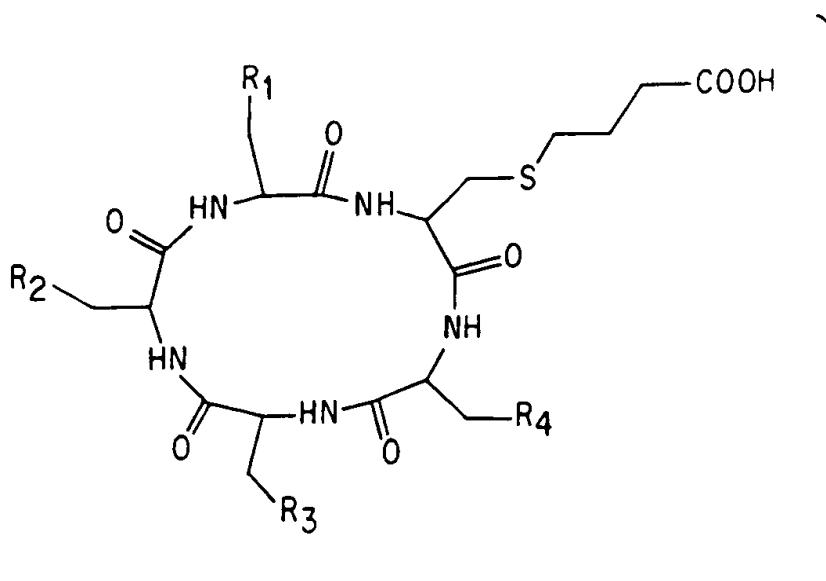
1. TG + Br-CH2CH2CH2COOH
2. + Boc-Cys(R)-OMe
3. TFA
4. Fmoc-Dab(Boc)-OH/DIC/HOBt
5. TFA
6. R-COOH/DIC/HOBt
7. Pip/DMF
8. Fmoc-Dab(Boc)OH/DIC/HOBt

.
.
.

n. NaOH
n+1. DIC/HOBt

F I G. 6

FIG. 7

NH-R
NH CO

GA,1

GI,2

GV,3

GL,4

GM,5

GN,6

AA,7

AI,8

AV,9

AL,10

AM,11

AN,12

IA,13

CH₃

II,14

CH₃

IV,15

IL,16

H OH

IM,17

O R
NH

IN,19

VA,19

VI,20

VV,21

VL,22

EtO

VM,23

CH₃O NH

VN,24

CH₃ NH

LA,25

O R
N

LI,26

LV,27

LL,28

LM,29

LN,30

MA,31 H

EtO

MI,32

CH₃O NH

MV,33

CH₃ NH

ML,34

R₁ S-R
R₂
NH
O

MM,35
R₁=R₂=H

MN,36
R₁=R₂=CH₃
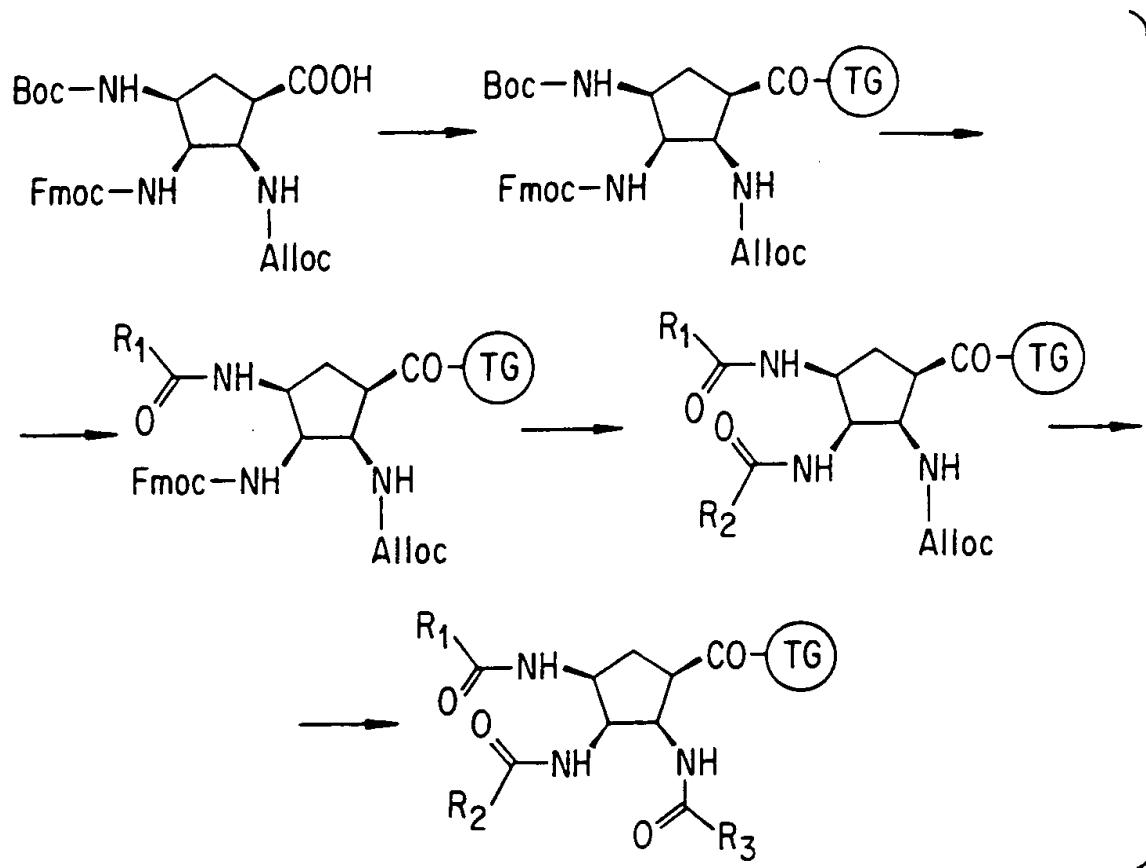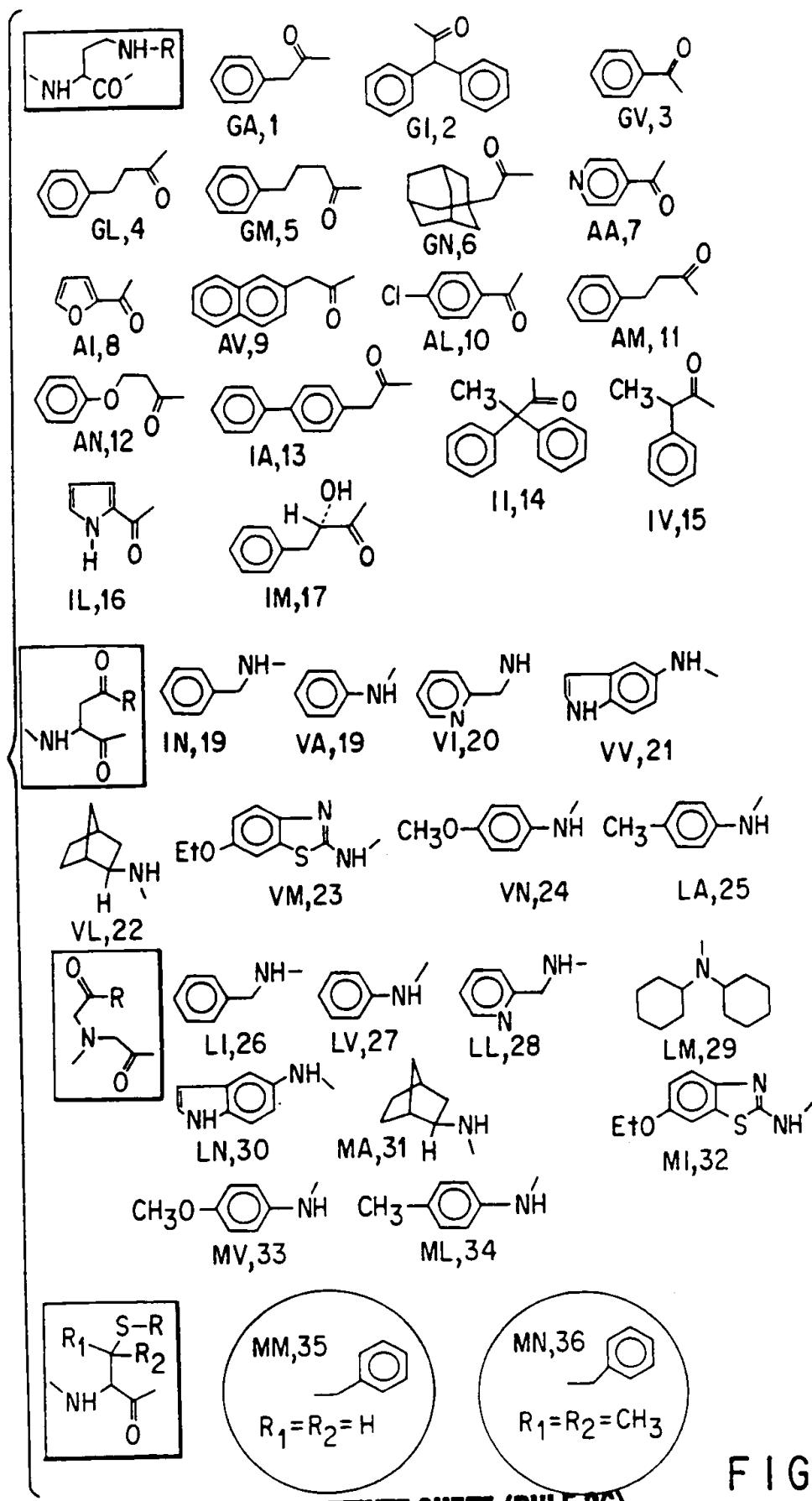
FIG. 8

SUBSTITUTE SHEET (RULE 26)

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For example, in polyamide synthesis, useful solid
phase support may be resins such as polystyrene (e.g.,
PAM-resin obtained from Bachem Inc., Peninsula
Laboratories, etc.), POLYHIPE® resin (obtained from
5   Aminotech, Canada), polyamide resin (obtained from
Peninsula Laboratories), polystyrene resin grafted
with polyethylene glycol (TentaGel®, Rapp Polymere,
Tubingen, Germany) or polydimethyl-acrylamide resin
(obtained from Milligen/Biosearch, California). In a
10 preferred embodiment for peptide and other polyamide
syntheses, the preferred solid phase support is
polydimethyl-acrylamide resin. Preferred solid phase
synthesis supports for specific syntheses are
described below. For example, in a model specific
15 embodiment, *infra*, a support such as is used in
Merrifield peptide synthesis can be sequentially
derivatized by an Fmoc-protected amino acid, which
through subsequent synthetic cycles is extended to
give the polyamide "synthetic test compound", and a Ddz-
20   or Boc-protected amino acid, which is extended to
give the coding peptide. Other sequential
derivatizations are described below. Thus, each resin
bead is functionalized to contain both synthetic test
compound and the corresponding coding structures, the
25 relative amounts of which depend on the reaction
conditions for attaching the first Fmoc- and Ddz- (or
Boc-) protected amino acids. In a variation of this
approach, the synthetic test compound and coding
molecules are attached to the solid support through
30 linkers such as those described below.
     The supports of the invention may also comprise
linkers or an arrangement of linkers. As used herein,
a linker refers to any molecule containing a chain of
atoms, e.g., carbon, nitrogen, oxygen, etc., that
35 serves to link the molecules to be synthesized on the

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support with the support. The linker is usually
attached to the support via a covalent bond, before
synthesis on the support starts, and provides one or
more sites for attachment of precursors of the
5   molecules to be synthesized on the support. Various
linkers can be used to attach the precursors of
molecules to be synthesized to the solid phase
support. Examples of linkers include aminobutyric
acid, aminocaproic acid, 7-aminoheptanoic acid,
10   8-aminocaprylic acid, lysine, iminodiacetic acid,
polyoxyethylene, glutamic acid, etc. In a further
embodiment, linkers can additionally comprise one or
more β-alanines or other amino acids as spacers.
         In another embodiment, the "safety-catch amide
15   linker" (SCAL) (see Patek, M. and Lebl, M. 1991,
Tetrahedron Letters 32:3891-3894; International Patent
Publication WO 92/18144, published October 29, 1992)
is introduced to the support.
         In addition to the linkers described above,
20   selectively cleavable linkers may be employed,
preferably for attachment of the synthetic test
compound molecule. One example is the ultraviolet
light sensitive linker, ONb, described by Barany and
Albericio (1985, J. Am. Chem. Soc. 107:4936-4942).
25   Other examples of photocleavable linkers are found in
Wang (1976, J.Org. Chem. 41:32-58), Hammer et al.
(1990, Int. J. Pept. Protein Res. 36:31-45), and
Kreib-Cordonier et al. (1990, in "Peptides -
Chemistry, Structure and Biology", Rivier and
30   Marshall, eds., pp. 895-897). Landen (1977, Methods
Enzym. 47:145-149) used aqueous formic acid to cleave
Asp-Pro bonds; this approach has been used to
characterize T-cell determinants in conjunction with
the Geysen pin synthesis method (Van der Zee et al.,
35   1989, Eur.J.Immunol. 191:43-47). Other potential

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linkers cleavable under basic conditions include those based on p-(hydroxymethyl)benzoic acid (Atherton et al., 1981, J. Chem. Soc. Perkin I:538-546) and hydroxyacetic acid (Baleaux et al., 1986, Int. J.

5 Pept. Protein Res. 28:22-28). Geysen et al. (1990, J. Immunol. Methods 134:23-33; International Publication WO 90/09395, published on August 23, 1990) reported peptide cleavage by a diketopiperazine mechanism. Preferred diketopiperazine linkages are disclosed in

10 U.S. Patent Application Serial No. 07/919,454, filed July 24, 1992, which is hereby incorporated by reference in its entirety. Enzyme-cleavable linkers may also be useful. An enzyme may specifically cleave a linker that comprises a sequence that is recognized

15 by the enzyme. Thus, linkers containing suitable peptide sequences may be cleaved by a protease and linkers containing suitable nucleotide sequences may be cleaved by an endonuclease. In certain instances, one may derivatize a portion (e.g., 10-90%) of the

20 available resin functional groups with a cleavable linker using certain reaction conditions, and the remaining of the resin functional groups with a linker which is stable to the cleavage conditions to ensure that enough material will remain on the resin after

25 cleavage for further study. This arrangement is particularly preferred when there is no coding molecule. Combinations of linkers cleavable under different reaction conditions can also be used to allow selective cleavage of molecules from a single

30 solid support bead.

    Preferably, a cleavable linker can be used to release the synthetic test compound, or a portion thereof, for testing in a screening assay. In this instance the coding sequence, if present, is attached

35 to the solid phase support via a non-cleavable linker.

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An approach for the synthesis of encoded
libraries involves linking the precursors of the
synthetic test compound and coding molecules of the
library together via a branched linker which also
5   serves to link both precursors to the solid support.
Depending on the structure of the linker, either
molecule or both may be detached from the solid
support for further study. One example of this
approach of anchoring synthetic test compound and
10 coding molecules is to use Lys(SCAL) derivatized
TentaGel.

A solid phase support linker for use in the
present invention may further comprise a molecule of
interest, which can be further derivatized to give a
15 molecular library. The pre-attached molecule may be
selected according to the methods described herein, or
may comprise a structure known to embody desired
properties.

The present invention encompasses the use of an
20 array of linkers attached to the solid support in one
of many arrangements. For example, a lysine carboxyl
group can be linked to a SCAL linker that is linked to
the solid phase support, thus producing a support
functionalized with a lysine-SCAL linker. In another
25 embodiment, a lysine can be linked to the solid phase
support via a polyethylene glycol linker. A SCAL
linker on a solid support can also be linked to an
amino group of a diamine linker, while the other amino
group can be used directly for further coupling. In
30 yet another embodiment, a cleavable linker can be
attached to one of the amino groups of a lysine linked
to a support while the other amino group is used
without further modification. Specific couplings to
each amino group of a lysine linker can be
35 accomplished by using orthogonal protecting groups.

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In a specific embodiment, *infra*, SCAL linked to
TentaGel can be acylated with lysine whose amino
groups are protected with Fmoc and Boc; the resulting
support is TentaGel with the linker Boc-Lys(Fmoc)-SCAL
5   which can be deprotected sequentially to provide two
unique amino groups which, e.g., upon acylations by
two different sets of amino acids, can become anchors
for two polyamides. Acylation of one of the amino
groups of the lysine moiety of the linker with Boc-
10 Lys(Fmoc) provides a new linker with a total of three
potential amino anchors (upon sequential
deprotection), and acylation of both amino groups of
the linker lysine by Boc-Lys(Fmoc) provides a new
linker with a total of four potential amino anchors.

15

5.3.2   TOPOLOGY OF ANCHORING OF SYNTHETIC TEST
        COMPOUNDS AND CODING MOLECULES ON SOLID
        SUPPORT SURFACES

A variety of approaches for topologically
separating the synthetic test compound and coding
20 molecules on a solid support in order to generate
libraries are contemplated.

Topologically separating the synthetic test
compound and the coding molecule refers to the
separation in space for a support. For example, if
25 the support is a resin bead, separation can be between
the surface and the interior of the resin bead of a
significant number of the ligand-candidate molecules
from a significant number of the coding molecules.
Preferably, the surface of the support contains
30 primarily synthetic test compound molecules and very
few coding molecules. More preferably, the surface of
the support contains greater than 90% synthetic test
compound. Even more preferably, the surface of the
support contains greater than 99% synthetic test
35 compound molecules; most preferably, it contains more

than 99.9% synthetic test compound. The advantage of
such an arrangement is that it limits interference of
the coding molecule in a binding screening assay (see
Section 5.6, *infra*). It is not necessary that the
5 topological area that contains the coding sequence,
i.e., the interior of a resin bead, be free of the
synthetic test compound.

In the foregoing example, the coding molecule is
segregated in the interior of the support particle.
10 It is also contemplated that the coding molecule may
be segregated to the surface of a support particle, or
to one side of a support particle.

One general approach for the topological
separation of synthetic test compound from coding
15 molecules involves the selective derivatization of
reactive sites on the support based on the
differential accessibility of the coupling sites to
reagents and solvents. For example, regions of low
accessibility in a resin bead are the interior of the
20 bead, e.g., various channels and other cavities. The
surface of a resin bead, which is in contact with the
molecules of the solution in which the bead is
suspended, is a region of relatively high
accessibility. Methods for effecting the selective
25 linkage of coding and synthetic test compound
precursors to a suitable solid phase support include,
but are not limited to, the following.

(i) Selective derivatization of solid support
surfaces via controlled photolysis: Two approaches
30 can be used. In one, a functionalized solid support
is protected with a photocleavable protecting group,
e.g., nitroveratryloxycarbonyl (Nvoc) (Patchornik et
al., 1970, J. Am. Chem. Soc. 92:6333). The Nvoc-
derivatized support particles are arranged in a
35 monolayer formation on a suitable surface. The

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monolayer is photolyzed using light of controlled
intensity so that the area of the bead most likely to
be deprotected by light will be the area of the bead
in most direct contact with the light, i.e., the
5   exterior surface of the bead. The resulting partially
deprotected beads are washed thoroughly and reacted
with a precursor of the synthetic test compound
containing a light-stable protecting group. For
example, in the case of synthesis of an encoded
10 library of polyamides, this precursor might be a Boc-
protected amino acid, which through further synthetic
cycles is converted to the polyamide synthetic test
compound. Following the reaction with the synthetic
test compound precursor, the beads are subjected to
15 quantitative photolysis to remove the remaining light-
sensitive protecting groups, thus exposing functional
groups in less light-accessible environments, e.g.,
the interior of a resin bead. After this quantitative
photolysis, the support particles are further
20 derivatized with an orthogonally-protected precursor
of the coding molecule, e.g., Fmoc-protected amino
acid. The resulting solid support bead will
ultimately contain synthetic test compound segregated
primarily on the exterior surface and coding molecules
25 located in the interior of the solid phase support
bead.
        An alternative photolytic technique for
segregating coding and synthetic test compound
molecules on a support involves derivatizing the
30 support with a branched linker, one branch of which is
photocleavable, and attaching the precursor of the
coding molecule to the photosensitive branch of the
linker. After completion of the synthesis, the
support beads are arranged in a monolayer formation
35 and photolyzed as described above. This photolysis

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provides beads which contain patches of synthetic test
compound molecules for selective screening with
minimal interference from the coding molecules.

(ii) Selective derivatization of solid support
5   surfaces using chemical or biochemical approaches.
The efficacy of these chemical and biochemical
derivatizations depends on the ability of exterior
surface functional groups, which are exposed, to react
faster than other groups in the interior which are not
10 exposed. It has been observed, for example, that
antibodies cannot bind to peptide ligands in the
interior of a resin solid phase support. Therefore,
using differences in steric hindrance imposed by the
structure of the support or by modulating the swelling
15 of a bead through choice of reaction solvent, reactive
groups on the exterior of the bead that are accessible
to macromolecules or certain reagents can be reacted
selectively with respect to reactive groups in the
interior of the bead. Therefore, the reactive groups
20 in the exterior of the bead can be modified for the
synthesis of the synthetic test compound, while
interior reactive groups can be modified for extension
of the coding molecules, or both the coding molecules
and synthetic test compound. Since the number of
25 reactive groups inside a resin bead is much larger
than the number of groups on the outer surface, the
actual number of coding molecules will be very large,
providing enough coding molecules for accurate
sequence analysis, and thus the decoding of the
30 structure of the synthetic test compound. A variety
of chemical and biochemical approaches are
contemplated including the following:

(a) Use of polymeric deprotecting agents to
selectively deprotect parts of the exterior of a solid
35 support bead carrying protected functional groups.

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The deprotected functional groups are used as anchors for the synthetic test compound. The functional groups which remain protected are subsequently deprotected using a nonpolymeric deprotecting agent

5   and used as anchors for the attachment of the coding molecules. In a specific embodiment, this method involves use of enzymes to selectively activate groups located on the exterior of beads which have been derivatized with a suitable enzyme substrate. Due to

10   their size, enzymes are excluded from the interior of the bead. In an example, *infra,* an enzyme completely removes a substrate from the surface of a resin bead, without significantly affecting the total amount of substrate attached to the bead, i.e., the interior of

15   the bead. The removal of substrate exposes, and thus activates, a reactive site on the bead. The enzyme-modified groups of the solid support are used to anchor the synthetic test compound and those groups that escaped modification are used to anchor the

20   majority of the coding molecules.

         (b) Use of a polymeric protecting group to selectively block exposed unprotected functional groups on the exterior of a support bead. The unprotected functional groups in the interior of the

25   support are used to anchor the coding molecule. The remaining protected functional groups are then deprotected and used as anchors for the synthetic test compound of the library. In a specific example, *infra,* the polymer polyglutamic acid of 30 kd MW

30   completely blocks surface accessible functional groups without affecting the total amount of peptide attached to the bead. If the polymer used for the blocking is attached via its α-carboxyl group, then a single-step Edman degradation performed after deprotection of the

35

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x-amino group of the polymer can regenerate the
surface amino groups.

        (c) Creating a different state in the
interior of the bead, e.g., by freezing water inside
the beads, then reacting the beads in an organic
solvent at low temperature to keep the water frozen.
Thus the surface of the bead, but not the inside, can
be specifically reacted.

10   5.4 STRATEGY FOR CARRYING OUT ALTERNATING
              SYNTHESES OF THE CODING AND SYNTHETIC TEST
              COMPOUNDS MOLECULES DURING THE GENERATION
              OF ENCODED LIBRARIES

An important synthetic operation during the
synthesis of an encoded library involves the use of
15 orthogonal protecting groups. For the efficient
synthesis of the coding molecules in parallel with the
synthesis of the synthetic test compound of the
library on the same solid support particle, the
protecting groups used for each synthesis must be
20 orthogonal, i.e., during all synthetic operations on
one molecule the protecting groups on the other
molecule must remain intact.

Several orthogonal combinations of protecting
groups for the assembly of the synthetic test compound
25 and coding molecules of a molecular library can be
used. Useful protecting groups are described in
Geiger and Konig, 1981, "The Peptides" (Gross and
Meinhofer, eds.) pp. 3-101, Academic Press: New York).
A very useful combination involves base- and acid-
30 cleavable protecting groups. For example, for the
synthesis of an encoded library of polyamides, the
base-sensitive N^{α}-[(9-fluorenylmethyl)oxy]carbonyl
(Fmoc-) protecting group may be used to assemble the
synthetic test compound molecules, and the acid-labile
35 N^{α}-[[2-(3,5-dimethoxyphenyl)prop-2-yl]oxy]carbonyl

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(Ddz) protecting group may be used to assemble the
coding peptide molecules. Fmoc protecting groups and
their use in peptide synthesis have been described by
Carpino and Han (1972, J. Org. Chem. 37:3403-3409) and
5   Ddz protecting groups have been described by Voss and
Birr (*Hoppe-Seyler's Z. Physiol. Chem. 1981, 362, 717-
725*). Both types of protecting groups have
traditionally been used to block the α-amino groups of
α-amino acids during peptide synthesis; however, other
10 suitable amino groups may be protected by these groups
during the synthesis of a polyamide. If the
polyamides of interest contain side-chains with
reactive functional groups, protection of the reactive
groups as *t*-butoxycarbonyl (Boc) and *t*-butyl (*t*-Bu)
15 derivatives, or preferably, as the more acid-stable
derivatives benzyl and benzyloxycarbonyl, may be
useful. If the reactive side-chain groups are
protected using *t*-butyl-type groups, the coding
peptide may be synthesized using a protecting group
20 which is more acid-labile than Ddz, such as Nps
(Zervas et al., 1963, J. Am. Chem. Soc. 85:3660) or
Trt (Zervas et al., 1956, J. Am. Chem. Soc. 78:1359)
An alternative combination of orthogonal
protecting groups in the synthesis of an encoded
25 library of polyamides involves use of Fmoc or other
base-labile groups to assemble the coding peptides and
Ddz or other acid-labile groups to assemble the ligand
binding candidates.
An alternative orthogonal combination of
30 protecting groups for the alternating and parallel
synthesis of coding molecules and synthetic test
compound involves trichloroethoxycarbonyl as an amine
protecting group and trichloroethyl as a hydroxyl
protecting group, which can be removed using a
35 reducing agent such as zinc in acetic acid, for the

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synthesis of, e.g., polyesters in an encoded library
of polyesters, and Boc and *t*-Bu or other acid-
cleavable group for the synthesis of the coding
peptides. As before, the two sets of orthogonal

5   protecting groups may be interchangeable, i.e., N^{α}-
trichloroethoxycarbonyl-protected amino acids are used
to prepare the coding peptides and N^{α}-Boc-protected
monomers are used to prepare the synthetic test
compound polyamides.

10   An additional useful combination of orthogonal
protecting groups involves the
trimethylsilylethoxycarbonyl group, which can be
removed by fluoride ions, and a highly acid-sensitive
protecting group such as Ddz or Bpoc (2-Biphenyl-2-

15   propoxycarbonyl). Either type of protecting group can
be used for N-protection during the assembly of either
the polyamide, in an encoded polyamide library
synthesis, or the coding peptide.

        For the synthesis of the peptide coding molecules
20   in preferred encoded libraries, the well-known
techniques of solid phase peptide synthesis including
suitable protecting group strategies will be used.
The relevant published art of peptide synthesis is
quite extensive and includes among others Stewart and

25   Young, 1984, "Solid Phase Synthesis", Second Edition,
Pierce Chemical Co., Rockford Il.; Bodanszky, Y.
Klausner, and M. Ondetti, "Peptide Synthesis", Second
Edition, Wiley, New York, 1976; E. Gross and J.
Meienhofer (editors), "The Peptides", vol. 1,

30   continuing series, Academic Press, New York, 1979.

            5.5 SPECIFIC LIBRARIES OF TEST COMPOUNDS
                 AND METHODS OF SYNTHESIS THEREOF
        Specific types of linkages for the libraries

35   listed in section 5 are described below as well as
synthetic reactions which can be used to generate

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these libraries, i.e., reactions that are used to
carry out step (ii) of the general procedure for
generating libraries (see section 5.2). As can be
readily appreciated by one of ordinary skill in the
5   art from the foregoing discussion and the following
exemplary material, any of the numerous condensation
reactions known in synthetic chemistry that can
proceed in a stepwise fashion with appropriate
protection groups can be used to prepare the libraries
10 of the invention. The list of subunits that can be
used to prepare such libraries is vast; many suitable
reagents can be obtained commercially or synthesized
using well known protocols. A partial list of
structures of suitable subunits is shown in Figure 5.
15 Examples of synthetic reactions are described in the
following subsections and Schema.

   In the Schemes herein, Z is any alkyl, aryl,
heteroalkyl or heteroaryl group, containing one or
more groups including but not limited to H, $-NH_2$, $-OH$,
20 CO_2H, $-CO_2R$, $-CONHR$, and the like. Alkyl means a C_1 to
C_{20} saturated or unsaturated hydrocarbon. Aryl means a
C_5 to C_{20} aromatic hydrocarbon. P with a circle refers
to a separate phase support, e.g., a resin bead. P
(without a circle) refers to a protecting group. The
25 remaining symbols have their standard meaning.

   These strategies can be employed to prepare
encoded libraries by using suitable orthoganol
protecting groups as described above.

30   5.5.1   LIBRARIES CONTAINING AMIDE BONDS
                     WITH SUBUNITS OTHER THAN α-AMINO
                     ACIDS
   A variety of libraries containing one or more
amide bonds, including libraries of polyamides whose
structures contain amino acids other than α-amino
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acids are contemplated. Scheme 1 shows a synthetic
strategy for polyamides:

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- 35 -

SCHEME I
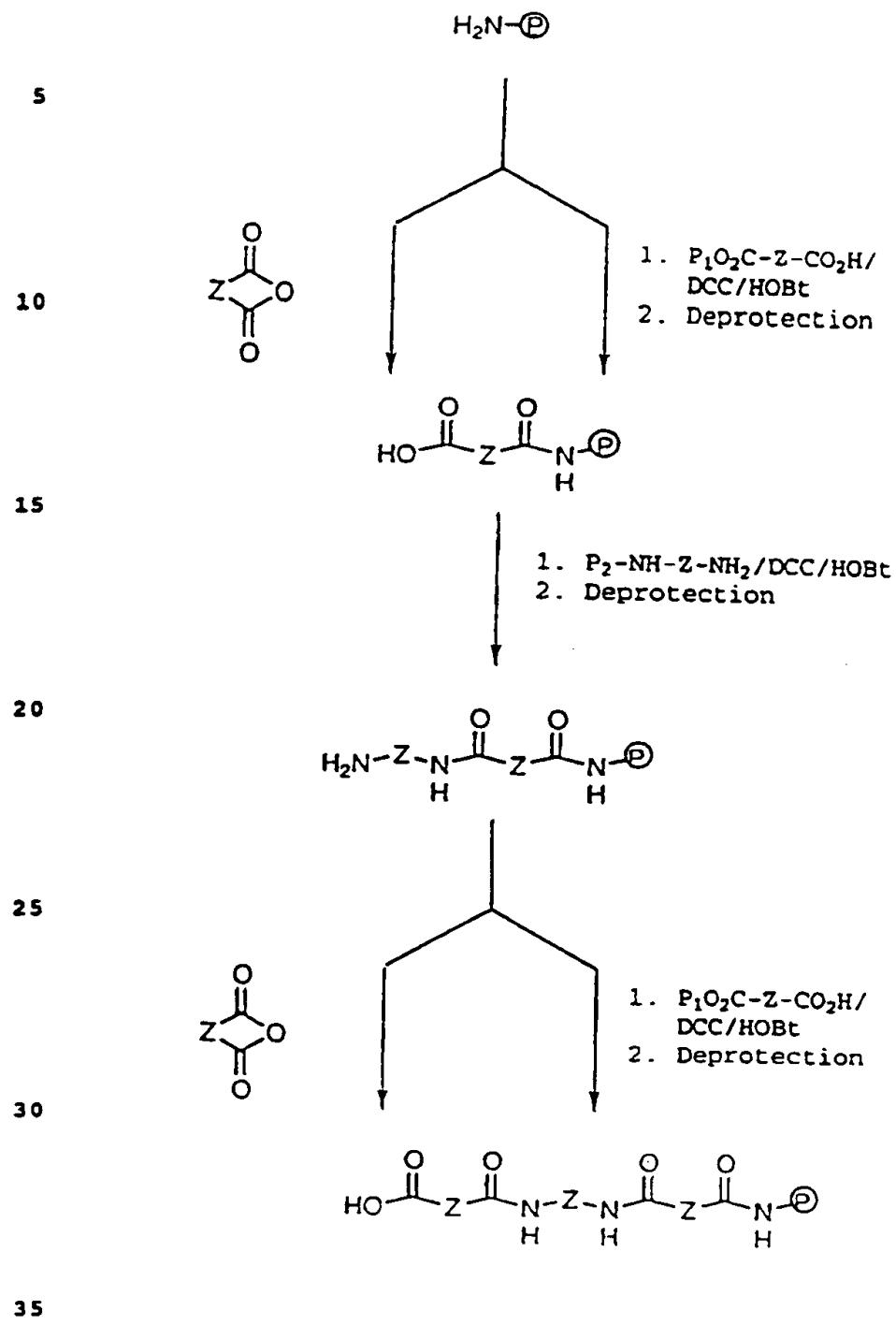
A suitable solid support, such as one of the supports described in section 5.2, is coupled with a carboxylic acid anhydride in a suitable solvent to give a carboxylic acid amide support. The supported

5   acid-amide is further elongated by activation of the carboxyl group, using a compound such as dicyclohexyl carbodiimide (DCC) in the presence of hydroxybenzotriazole (HOBt), followed by condensation with a diamine containing a protected amino group;

10 deprotection of the condensation product gives a diamide-amine on the solid support. Repetition of this synthetic cycle, i.e, sequential reaction with an anhydride, condensation with a single protected diamine, and deprotection, produces a growing

15 polyamide on the solid support. If the completed polyamide sequence contains protecting groups, it is deprotected without detachment from the solid support.

An alternative synthetic approach to polyamides involves modification of the above synthesis by

20 replacement of the anhydride with a partially protected dicarboxylic acid, e.g., a suitable dicarboxylic acid half ester. The resulting ester amide resin is deprotected and activated with, e.g., DCC/HOBt prior to condensation with the diamine.

25 For the synthesis of synthetic test compound polyamides whose structures contain α-amino acids, such as peptides and peptide mimetics, the peptide synthesis techniques described previously may be used.

In one embodiment, pyroglutamate may be included

30 as the N-terminal residue of the synthetic test polyamides of the library.

In a further embodiment, subunits that confer useful chemical and structural properties will be chosen for incorporation into a synthetic test

35 polyamide sequence. In particular, the present

invention envisions preparing libraries of polyamides
that have more well defined structural properties than
native peptides. In another embodiment, a polyamide
library may be generated that incorporates a reduced

5   peptide bond, i.e., $R_1-CH_2-NH-R_2$, where R_1 and R_2 are
amino acid residues or sequences. A reduced peptide
bond may be introduced as a dipeptide subunit. Such a
molecule would be resistant to peptide bond
hydrolysis, e.g., protease activity. Such libraries

10 could provide ligands with unique function and
activity compared with those of the corresponding
native peptides, such as extended half-lives *in vivo*
due to resistance to metabolic breakdown, or protease
activity.

15   A constrained, cyclic or rigid polyamide may be
prepared according to the method described previously,
provided that, in at least two positions in the
sequence of all synthetic test compound, subunits,
e.g., amino acids, are inserted that provide chemical

20 functional groups capable of crosslinking to
constrain, cyclize or make rigid the polyamide after
treatment to form a crosslink. Examples of amino
acids capable of crosslinking a peptide are cysteine
to form disulfides, aspartic acid to form a lactone or

25 a lactam, and a chelator such as γ-carboxyl-glutamic
acid (Gla) (commercially available, e.g., from Bachem)
to chelate a transition metal and form a cross-link.
Protected γ-carboxyl glutamic acid may be prepared by
modifying the synthesis described by Zee-Cheng and

30 Olson (1980, Biophys. Biochem. Res. Commun. 94:1128-
1132). A library in which the polyamide sequence
comprises at least two subunits capable of
crosslinking may be treated, e.g., by oxidation of
cysteine residues to form a disulfide or addition of a

35 metal ion to form a chelate, so as to crosslink the

- 38 -

peptide and form a constrained, cyclic or rigid
peptide. Cyclic motifs are disclosed in detail in
U.S. Application Serial no. 07/717,454, filed June 19,
1991.

5   Some simple amino acids that can be used as
subunits for incorporation into a library include the
following:

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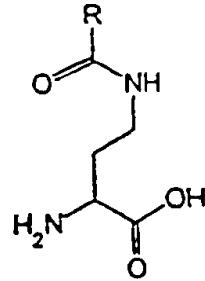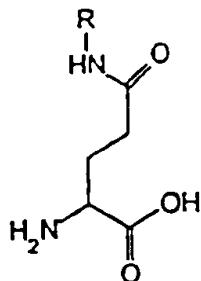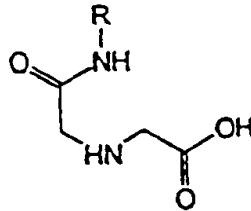
15

Non-classical amino acids may be used during the
synthesis of polyamides. The following non-classical
amino acids may be incorporated in a polyamide library
20 in order to introduce particular conformational
motifs: 1,2,3,4-tetrahydroisoquinoline-3-carboxylate
(Kazmierski et al., 1991, J. Am. Chem. Soc. 113:2275-
2283); (2S,3S)-methyl-phenylalanine, (2S,3R)-methyl-
25 phenylalanine, (2R,3S)-methyl-phenylalanine and
(2R,3R)-methyl-phenylalanine (Kazmierski and Hruby,
1991, Tetrahedron Lett.); 2-
aminotetrahydronaphthalene-2-carboxylic acid (Landis,
1989, Ph.D. Thesis, University of Arizona); hydroxy-
30 1,2,3,4-tetrahydroisoquinoline-3-carboxylate (Miyake
et al., 1984, J. Takeda Res. Labs. 43:53-76); β-
carboline (D and L) (Kazmierski, 1988, Ph.D. Thesis,
University of Arizona); HIC (histidine isoquinoline
carboxylic acid) (Zechel et al., 1991, Int. J. Pep.
35 Protein Res. 38:131-138).

The following amino acid analogs and
peptidomimetics may be incorporated into the library
of synthetic test compound to induce or favor specific
secondary structures: LL-Acp (LL-3-amino-
5   2-propenidone-6-carboxylic acid), a β-turn inducing
dipeptide analog (Kemp et al., 1985, J. Org. Chem.
50:5834-5838); β-sheet inducing analogs (Kemp et al.,
1988, Tetrahedron Lett. 29:5081-5082); β-turn inducing
analogs (Kemp et al., 1988, Tetrahedron Lett. 29:5057-
10   5060); α-helix inducing analogs (Kemp et al., 1988,
Tetrahedron Lett. 29:4935-4938); γ-turn inducing
analogs (Kemp et al., 1989, J. Org. Chem. 54:109:115);
and analogs provided by the following references:
Nagai and Sato, 1985, Tetrahedron Lett. 26:647-650;
15   DiMaio et al., 1989, J. Chem. Soc. Perkin Trans.
p. 1687; also a Gly-Ala turn analog (Kahn et al.,
1989, Tetrahedron Lett. 30:2317); amide bond isostere
(Jones et al., 1988, Tetrahedron Lett. 29:3853-3856);
tretrazol (Zabrocki et al., 1988, J. Am. Chem. Soc.
20   110:5875-5880); DTC (Samanen et al., 1990, Int. J.
Protein Pep. Res. 35:501-509); and analogs taught in
Olson et al., 1990, J. Am. Chem. Soc. 112:323-333 and
Garvey et al., 1990, J. Org. Chem. 56:436.
     The present invention further provides for
25   modification or derivatization of synthetic test
compound polyamides in a library such as described in
U.S. Application Serial No. 07/717,454, filed June 19,
1991. Modifications of peptides are well known to one
of ordinary skill, and include phosphorylation,
30   sulfation, carboxymethylation, and acylation.
Modifications may be effected by chemical or enzymatic
means. Since such modifications may result in non-
sequenceable peptides, use of a coding molecule in
such libraries is preferred.

35

- 40 -

In another aspect, glycosylated or fatty acylated peptide derivatives may be prepared. Preparation of glycosylated or fatty acylated peptides is well known in the art (see, e.g., U.S. Application Serial No.
5 07/717,454).

Fatty-acid polyamide derivatives may also be prepared. For example, and not by way of limitation, a free amino group may be acylated, e.g., myristoylated. This and other peptide-fatty acid
10 conjugates suitable for use in the present invention are disclosed in U.K. Patent GB-8809162.4, International Patent Application PCT/AU89/00166.

5.5.2 LIBRARIES CONTAINING CARBAMATE BONDS
15   The present invention encompasses synthetic test compounds that include one or more carbamate (i.e., polyurethane) bonds, including polycarbamates. Two strategies for forming carbamates are shown in Scheme II.
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SCHEME II

- 42 -
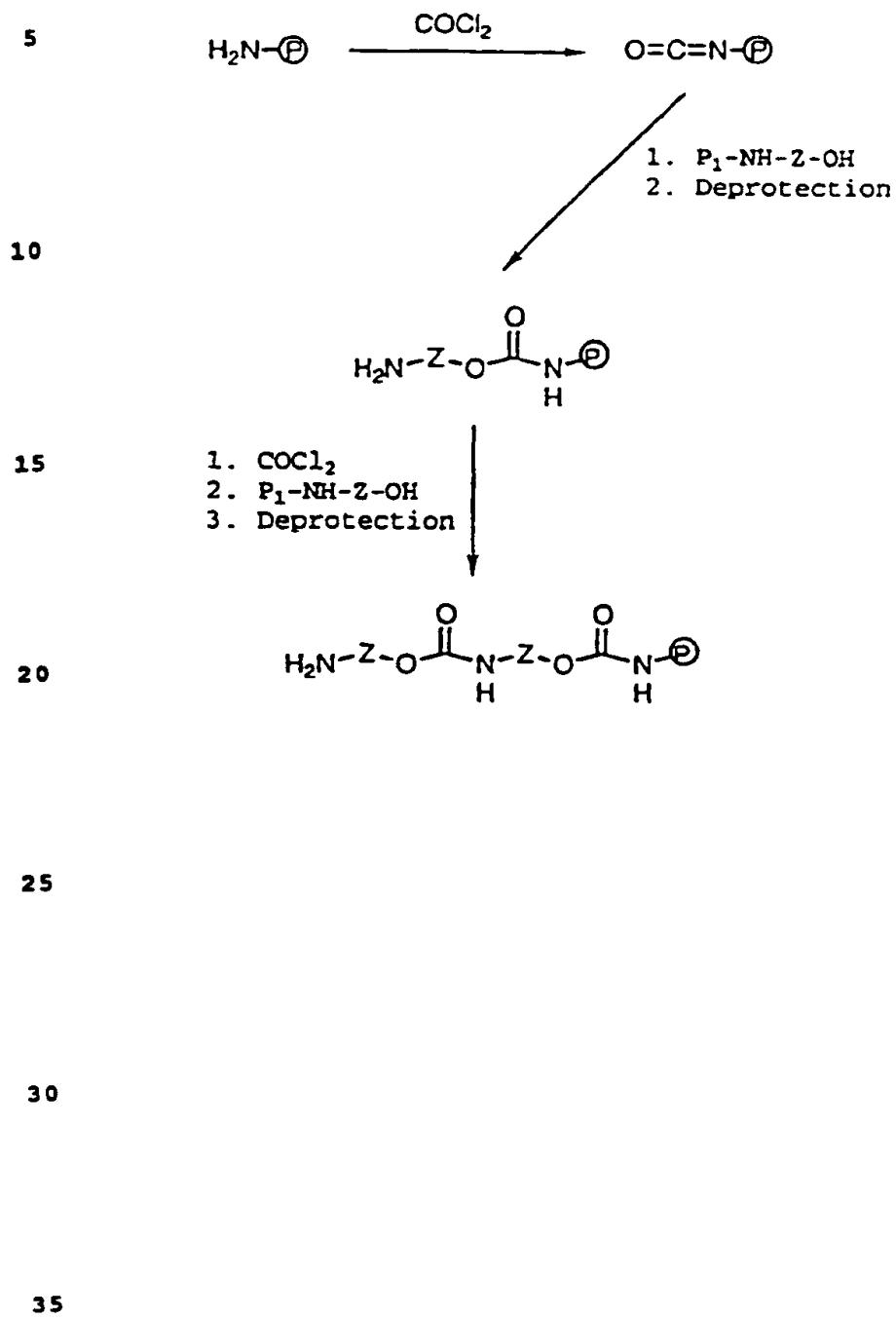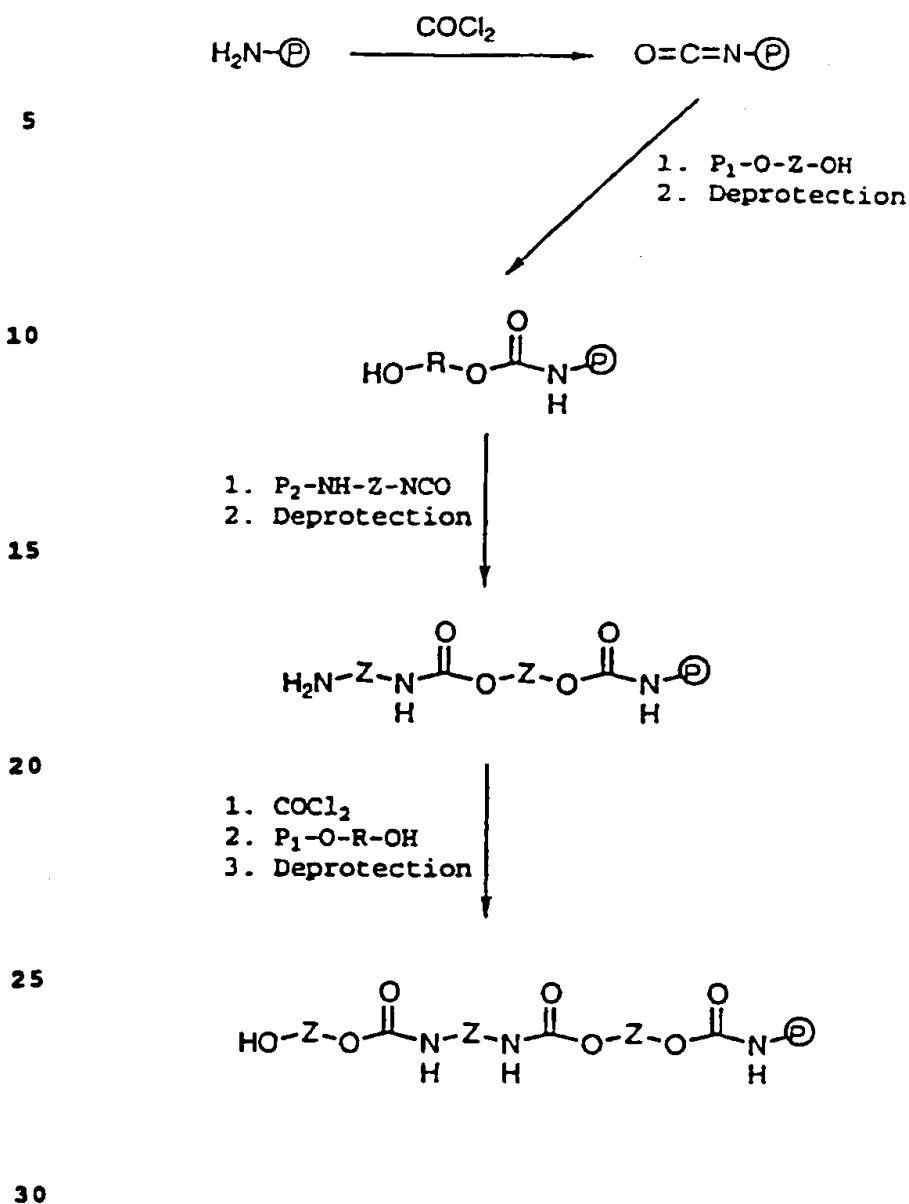
- 43 -

For the synthesis of the two different types of carbamates, couplings of isocyanates and diols or aminoalcohols can be used. For example, a suitable resin, such as the functionalized resin used for the
5   synthesis of polyamides above, is converted to the isocyanate by reaction with phosgene, and the isocyanate is then coupled with an amino-protected aminoalcohol to give the protected carbamate. Deprotection of the resin urethane furnishes an
10 aminourethane resin which is used to repeat the same synthetic cycle producing a polyurethane, i.e., reaction with phosgene followed by coupling with an N-protected aminoalcohol and deprotection produces an amino diurethane resin, etc.

15   A second type of supported carbamate is produced by modifying the above synthetic procedure as follows. The starting amino resin is converted to the isocyanate, the isocyanate is converted to a protected carbamate by reaction with partially protected diol,
20 the protected carbamate resin is deprotected to give a hydroxycarbamate, the hydroxycarbamate is converted to an aminodicarbamate by reaction with an amino-protected aminoalkylisocyanate, which is followed by deprotection.

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5.5.3   LIBRARIES CONTAINING UREA BONDS
A strategy for synthesis of variety of urea-bond containing structures is shown in Scheme III.

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- 44 -

SCHEME III
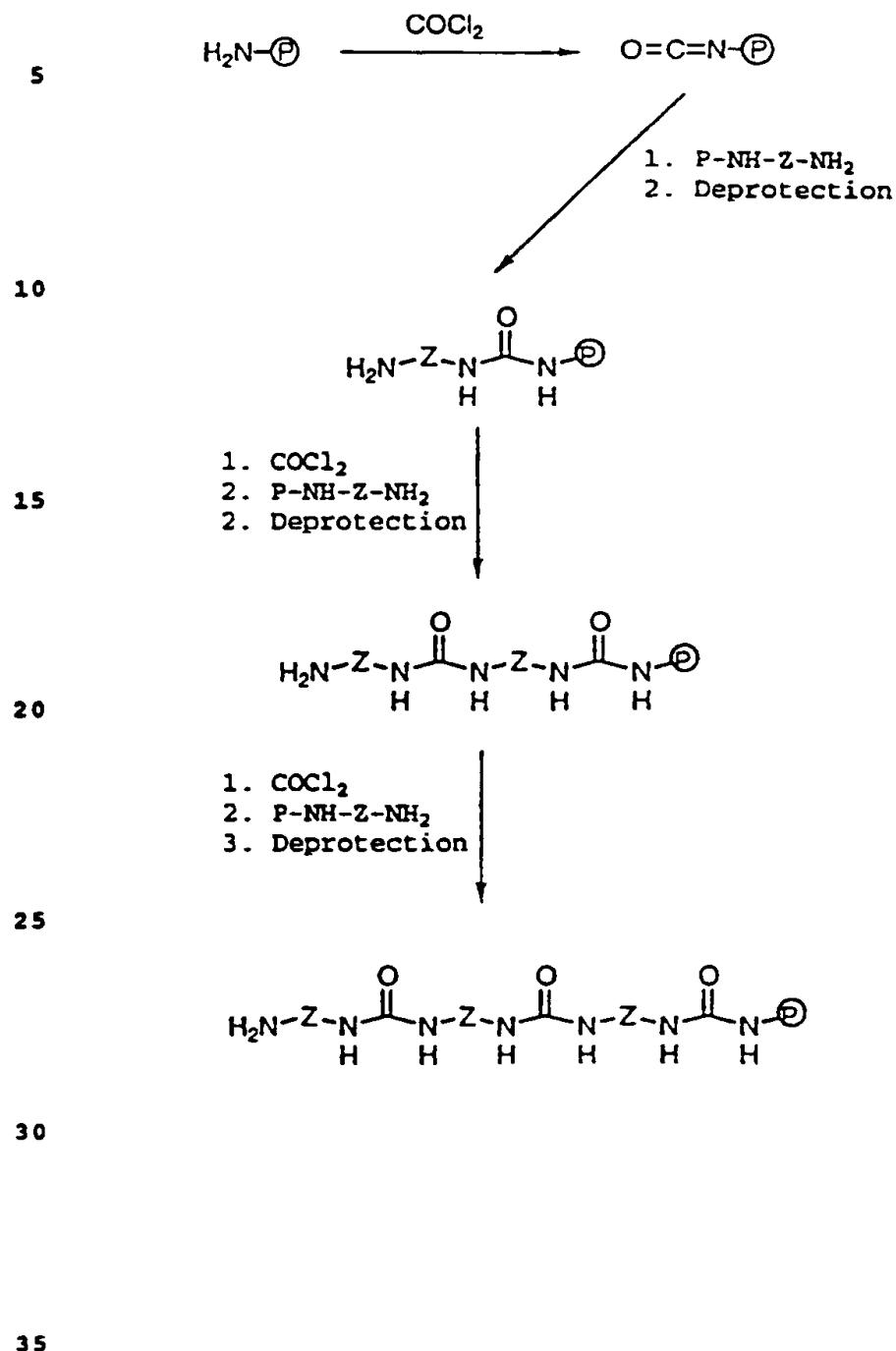
- 45 -

A suitable resin functionalized with isocyanate
groups, such as the resin used in the synthesis of the
carbamates described above, is converted to an amino
urea by reaction with a partially protected diamine
5   followed by deprotection, and the amino urea resin to
an isocyanate using phosgene. The isocyanatourea
resin is subjected to the above three-step synthetic
cycle the desired number of times producing polyureas.

10   5.5.4 LIBRARIES CONTAINING ESTER BONDS
A strategy for the synthesis of libraries
containing ester bonds is shown in Scheme IV.

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SCHEME IV
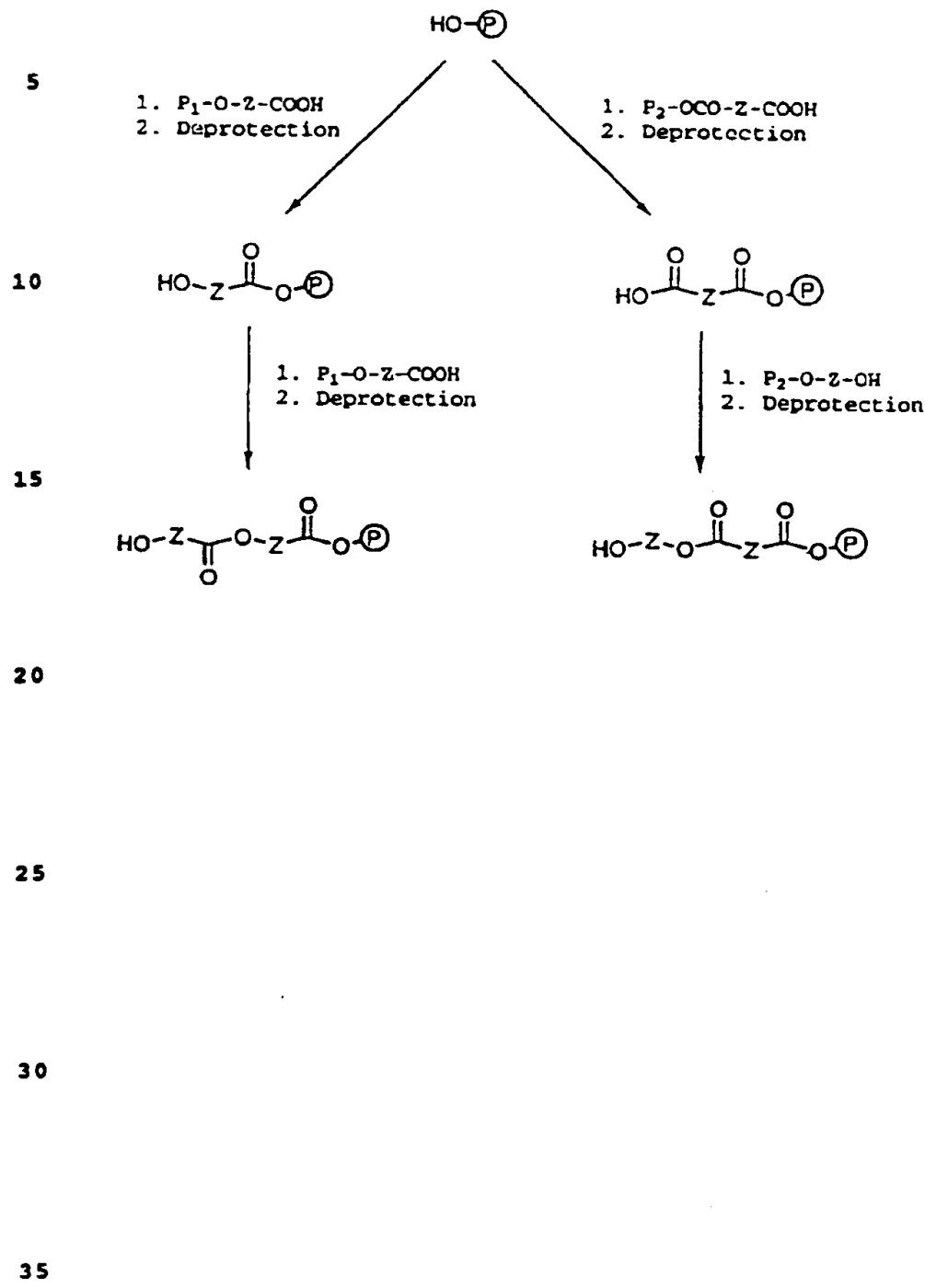
A suitable resin, such as the hydroxyalkyl resin used in Merrifield solid phase peptide synthesis, in a swelling solvent such as methylene chloride, is condensed with a suitably protected hydroxycarboxylic
5   acid, preferably in the presence of a condensing agent such as DCC, to give, after deprotection, a supported hydroxyester which is further elongated using the same acylation-deprotection cycles.

10   5.5.5   LIBRARIES CONTAINING AMINE BONDS
A strategy for the synthesis of amines is shown in scheme V:

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SCHEME V
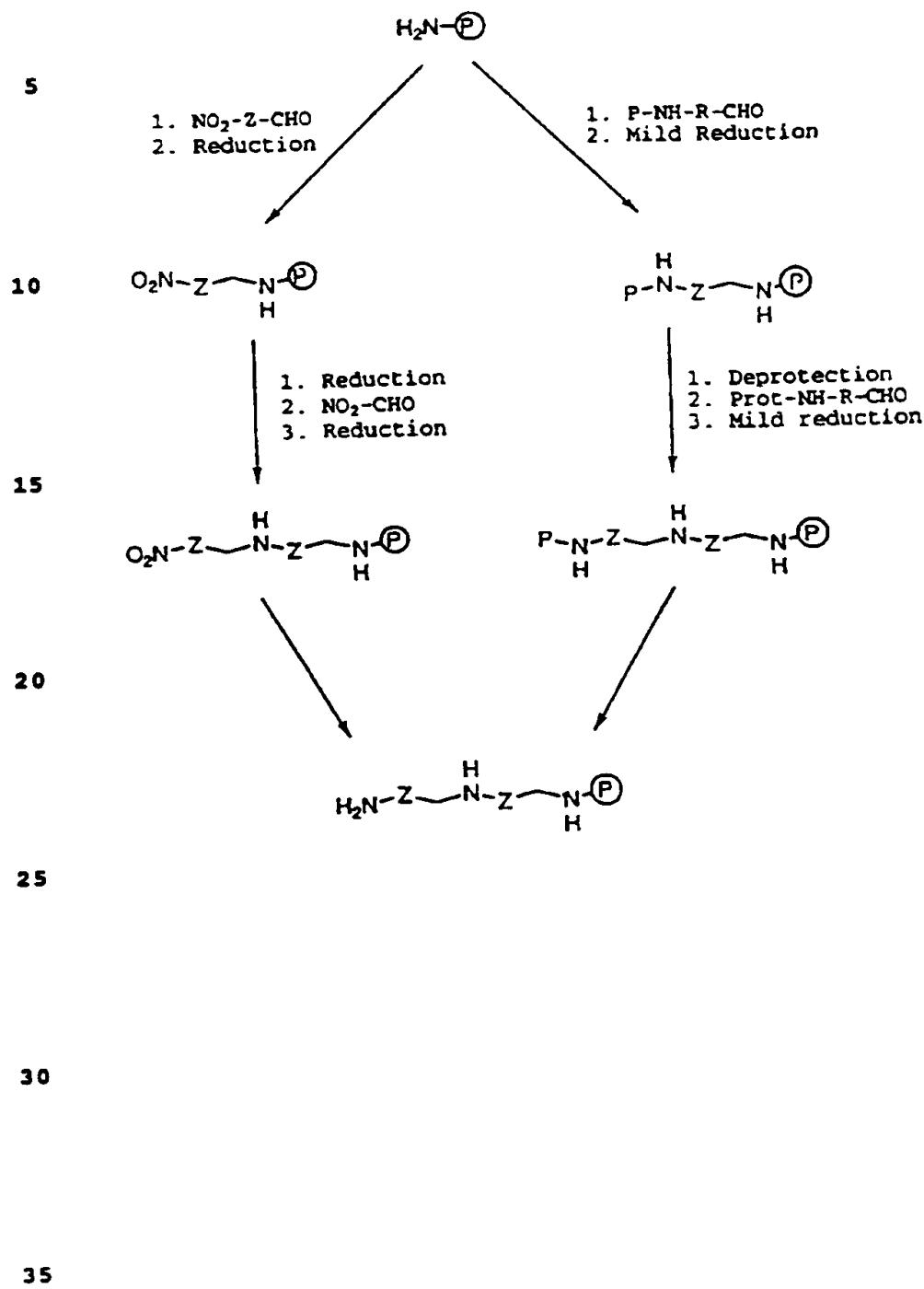
- 49 -

A suitable resin, such as the resin used in the
amide synthesis above, is converted to a nitroalkyl
amine resin via reductive amination using a
5   nitroaldehyde, and further reduced to the primary
amine, using one of many reactions known to reduce
nitroalkamines to primary amines (e.g., reduction by
lithium aluminum hydride). The primary amine on the
resin is elongated by repeating the reductive-
10   alkylation-reduction sequence, producing the desirable
polyamine.

The reduction of a nitroalkylamine resin in the
procedure above can be avoided by replacing the
nitroaldehyde of the reductive amination with an N-
15   protected aminoaldehyde and removing the protecting
group of the resulting product in a separate synthetic
step.

5.5.6   LIBRARIES CONTAINING
           SULFIDES AND DISULFIDE BONDS
20
A strategy for synthesis of a variety of
polysulfide and polydisulfide structures is shown in
scheme VI:

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- 50 -

SCHEME VI

5

HS-(P)

1. P_1-S-Z-L
2. Deprotection

1. P_2-S-Z-S-Act
2. Deprotection

10

HS-Z-S-(P)   HS-Z-S-S-(P)

1. P_1-S-Z-L
2. Deprotection

1. P_2-S-Z-S-Act
2. Deprotection

15

HS-Z-S-Z-S-(P)   HS-Z-S-S-Z-S-S-(P)

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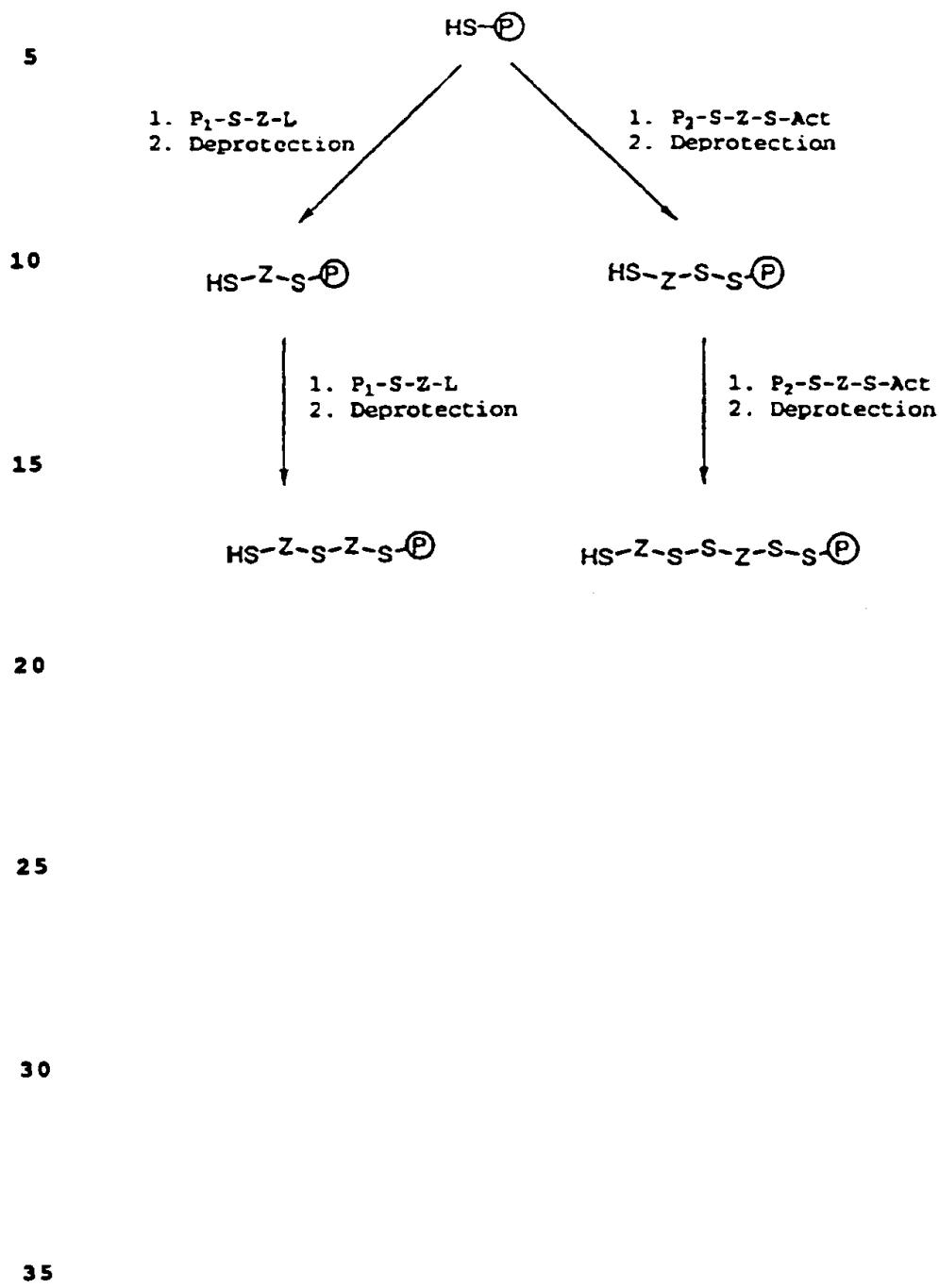
- 51 -

A suitable resin, e.g., a resin used in
Merrifield solid phase peptide synthesis, is
functionalized to have free thiol groups. The thiol
resin is alkylated with a protected thioalkyl halide
5   and the product deprotected to give a thioalkyl resin
sulfide. The supported thioalkylsulfido chain is
further elongated by repeating the alkylation-
deprotection cycle to furnish a supported polysulfide.
The above synthesis can yield supported
10   disulfides if the protected thioalkyl halide is
replaced by a protected thioalkylchlorosulfenate or
thioalkylmethoxycarbonylsulfenate.

    5.5.7   **LIBRARIES CONTAINING CARBON-CARBON
               BONDS**
15
    A variety of polyalkanes, polyalkenes,
polyhaloalkene and, polyols are contemplated.
Strategies for the synthesis of such libraries are
shown in Scheme VII:

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- 52 -

SCHEME VII
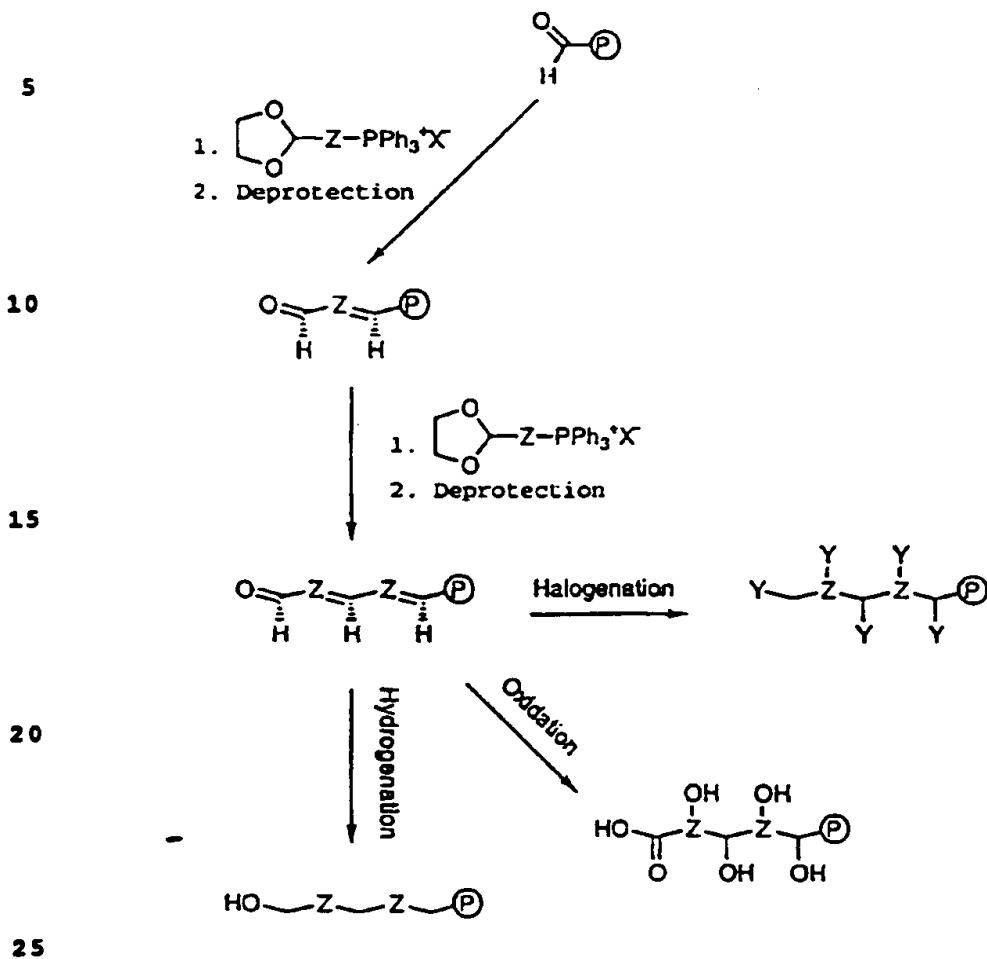
- 53 -

A suitable resin is functionalized with carbonyl groups, (e.g., by controlled oxidation of support hydroxyalkyl groups), condensed with a Wittig reagent, prepared from triphenyl phosphine and a haloalkyl

5 dialkyl acetal, and deprotected to give a resin containing an unsaturated aldehyde chain. This chain can be elongated to a polyene aldehyde using the same Wittig-condensation-deprotection sequence. Treatment of the supported polyene with a molecular halogen,

10 such as chlorine or bromine produces a haloalkane on a resin. Conversion of these haloalkanes to their fully dehalogenated reduced derivatives by reaction with tributyl tin hydride or an electropositive metal such as zinc in the presence of a weak acid is also

15 contemplated. Careful treatment of the supported polyene with an oxidizing agent, e.g., permanganate or periodate, produces polyols. Other polyols may be produced by subjecting the polyene to a hydroboration-oxidation sequence, an epoxidation (by a peracid such

20 as m-chloroperbenzoic acid)-hydrolysis sequence, or a mercuric-acetate-alkaline-borohydride sequence.

5.5.8   LIBRARIES OF POLYCYCLIC COMPOUNDS AND FUNCTIONALIZED POLYCYCLIC COMPOUNDS

25   A variety of polycyclic and functionalized polycyclic compounds are contemplated. A strategies for the synthesis of polycyclic and related structures are shown in Scheme VIII, in which R_1, R_2 and R_3 refer various substituted alkyl or aryl groups as defined

30 above:

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- 54 -

SCHEME VIII

- 55 -
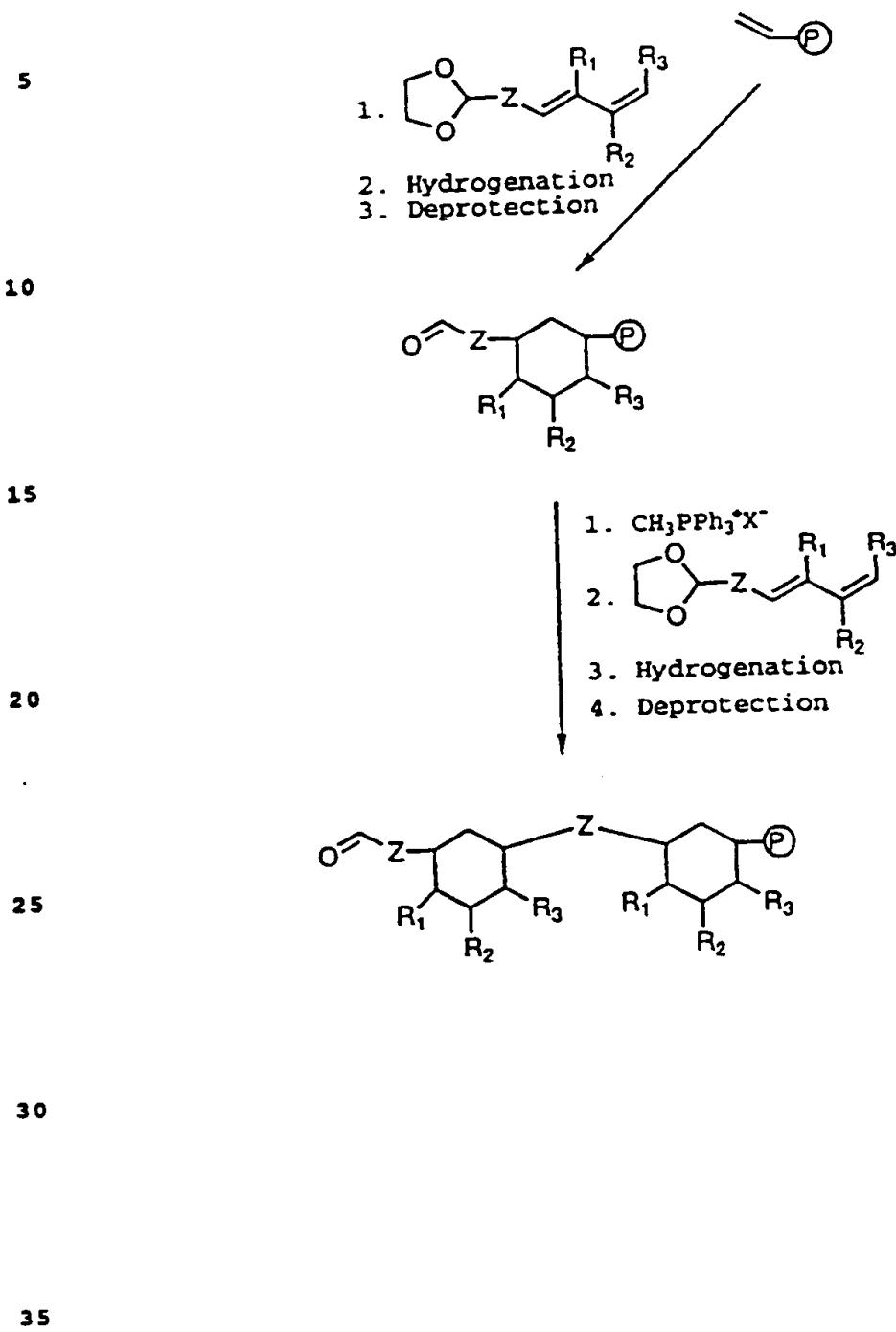
Scheme VIII shows preparation of a saturated hydrocarbon. Alternatively, unsaturated structures can also be prepared. A suitable resin, such as the carbonyl resin described above, is converted to an

5 alkene resin, e.g., by a Wittig condensation, and this resin is used in a Diels-Alder-type pericyclic reaction with a suitably activated, e.g., electrophilic, diene dimethylacetal to produce, after hydrolysis of the acetal group, a supported

10 functionalized cyclohexenyl aldehyde. This supported structure can be further elongated by repetition of the Wittig-condensation-Cycloaddition-deprotection sequence producing a supported poly-cyclohexene aldehyde which can be further functionalized as

15 follows (i) by halogenation, e.g., bromination or chlorination, to give a poly-halocyclohexanyl aldehyde, (ii) by reduction of the poly-halocyclohexanyl aldehyde, e.g., using tributyltin hydride or an electropositive metal (e.g., Zn) and a

20 weak acid, producing a polycyclohexane aldehyde or alcohol, and (iii) by controlled oxidation of the polycyclohexane aldehyde using permanganate, a hydroboration-oxidation sequence, an epoxidation-hydrolysis sequence, or an epoxidation-reduction

25 sequence (e.g., epoxidation by m-chloroperbenzoic acid followed by reduction by lithium aluminum hydride) producing a functionalized polyol.

Another example of a cyclic library is shown in Figure 6. This library is prepared by attaching

30 bromo-propionic acid to a support, and attaching Boc-protected cysteine methyl ester to the support via substitution of bromine with the sulfur side chain to form a sulfide linkage. Boc/Fmoc protected diamino acids, such as diaminobutyric acid or lysine, can be

35 added. Deprotection of one protecting group allows

- 56 -

substitution with any carboxylic acid. Deprotection
of the other amino group allows addition of another
diamino acid. This sequence of steps is repeated the
desired number of times. Finally, the methyl ester
5 group is hydrolyzed from cysteine, allowing reaction
with a deprotected amino group to cyclize the
structure.

       5.5.9   LIBRARIES OF POLYSUBSTITUTED RING
                 STRUCTURES CAPABLE OF SERVING AS A
10   SCAFFOLDING

A variety of polysubstituted structures capable
of serving as a scaffolding are contemplated. A
general strategy for the synthesis of such structure
is shown in Scheme IX:
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- 57 -

SCHEME IX
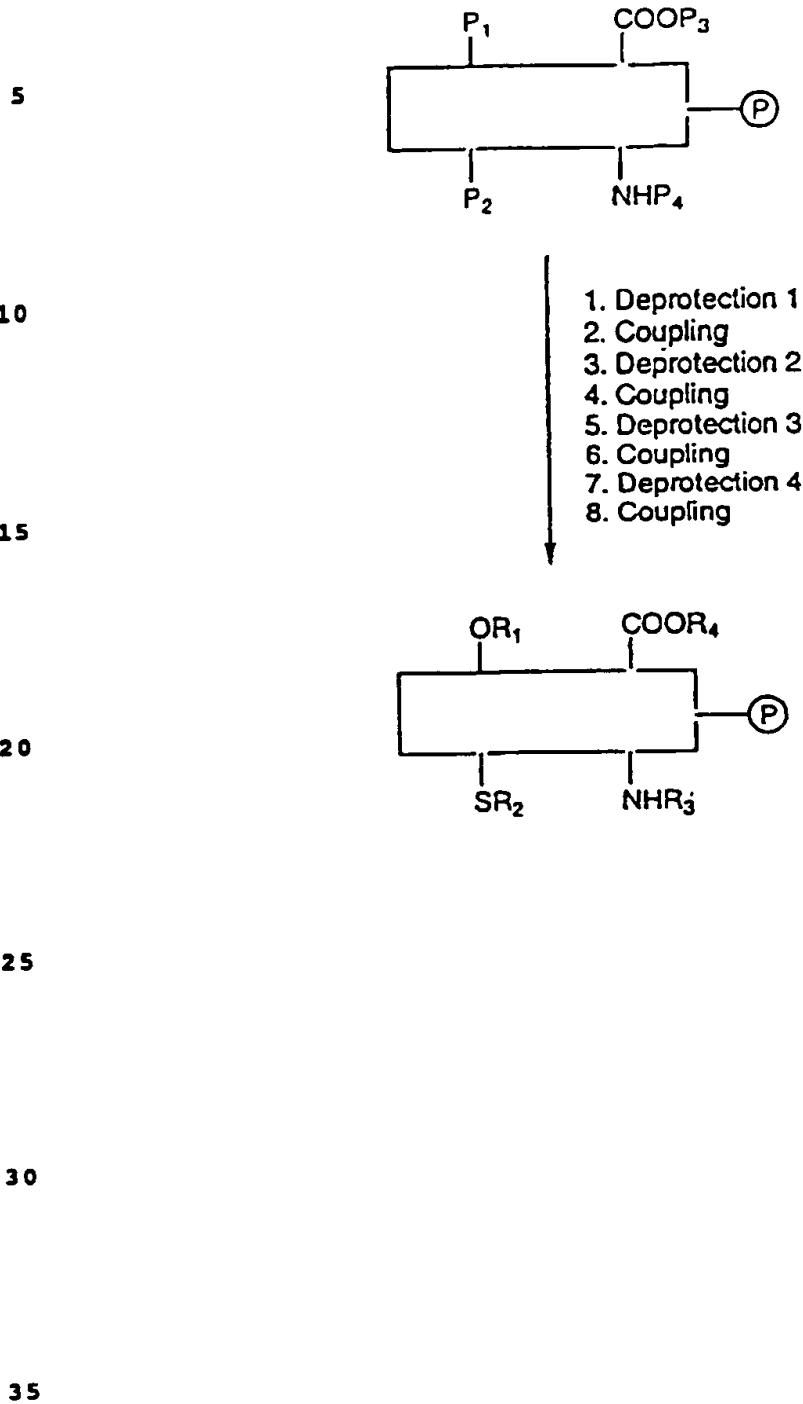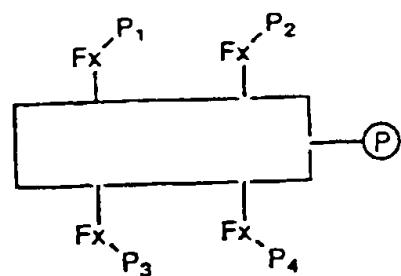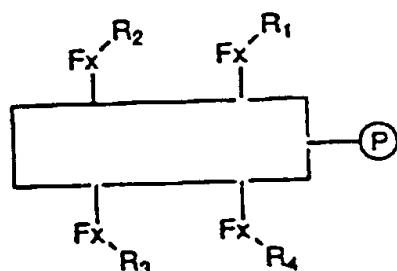
- 59 -

A general strategy for preparation of a specific
type of scaffold libraries is shown in scheme X:

SCHEME X
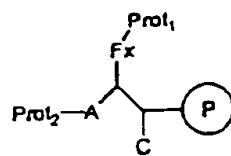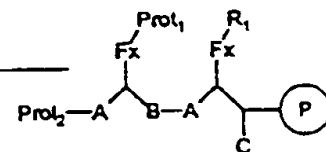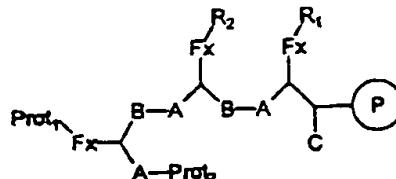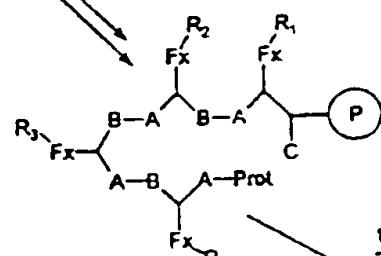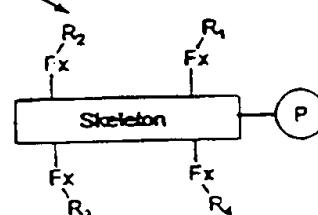
- 60 -

Suitable scaffolds include but are not limited to
cyclopentane, Kemp's triacid (Kemp and Petrakis, 1981,
J. Org. Chem. 46:5140-5143), a branched construct
prepared by consecutive coupling of diamino carboxylic
5 acids, cyclic templates such as are described by
Mutter, et al. (1992, J. Am. Chem. Soc. 114-1463-
1470), steroids, benzodiazepines, and the like.

An approach for the attachment of a derivative of
cis-1,3,5-cyclohexane tricarboxylic acid to a
10 Merrifield-type resin followed by derivatization of
each of the three carboxyl groups of the triacid is
described in Section 9, infra, as one example of a
synthesis of a supported polysubstituted ring
structure capable of serving as a scaffolding (see
15 Scheme XIV, infra).

As a second example of a synthesis of a
polysubstituted ring structure capable of serving as a
scaffolding, the assembly and derivatization of 1,4-
benzodiazepines, based on the published work of Ellman
20 and Bunin (Chemical & Engineering News, Jan. 18, 1993,
p. 33), is given below. In a specific embodiment, a
suitable resin, such as a Merrifield-type resin
functionalized with a 4-hydroxymethylphenoxyacetic
acid linker, is further functionalized with a 2-amino-
25 4'-hydroxybenzophenone, whose amino group is protected
with the fluorenylmethoxycarbonyl (Fmoc) group. After
removal of Fmoc, coupling of the resulting aniline
with an Fmoc-protected amino acid, to produce an
anilide, removal of Fmoc from the anilide and
30 cyclization, a supported 1,4-benzodiazepine is
produced which can be further alkylated on the anilide
nitrogen, using a variety of electrophiles, to produce
a variety of benzodiazepine derivatives for further
study.

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- 61 -

One of the smallest possible ring structures for use as a scaffold is the cyclopentane ring (Figure 7).

Encoded versions of the polysubstituted ring structures libraries described above are preferred
5   embodiments. Encoded libraries in which the coding molecules are peptides are most preferred. For the synthesis of the peptide-encoded libraries the general procedure of section 5.1 and the synthetic strategies outlined in sections 5.2 and 5.3 are used.
10

5.5.10   LIBRARIES BASED ON SCAFFOLDING
            CONSTRUCTED FROM AMINO ACIDS

A scaffolding, mapping larger conformational space, that is a simple branched attachment, is
15   constructed by consecutive coupling of diamino carboxylic acids (see Figure 3). Various types of the scaffolding mapping extensive space are flexible cyclic or branched scaffoldings. The principles of these libraries are illustrated in Figure 3. As an
20   example of scaffold library construction we show here synthesis of branched library (Scheme XV, Section 9, infra).

5.6 METHODS OF DETECTION AND IDENTIFICATION
        OF LIGANDS IN LIBRARIES OF TEST COMPOUNDS
25   In addition to providing libraries of a great variety of chemical structures as synthetic test compound, and methods of synthesis thereof, the present invention further comprises methods of screening the test compounds of a library to identify
30   ligands within the library that demonstrate a biological activity of interest, such as binding, stimulation, inhibition, toxicity, taste, etc. Other libraries may be screened according to the methods described infra for enzyme activity, enzyme inhibitory
35   activity, and chemical and physical properties of

- 62 -

interest. Many screening assays are well known in the
art; numerous screening assays are also described in
U.S. Patent Application Serial No. 07/717,454, filed
June 19, 1991).

5   The ligands discovered during an initial
screening may not be the optimal ligands. In fact, it
is often preferable to synthesize a second library
based on the structures of the ligands selected during
the first screening. In this way, one may be able to
10   identify ligands of higher activity.

        5.6.1 BINDING ASSAYS
        The present invention allows identification of
synthetic test compound that bind to acceptor
15   molecules. As used herein, the term "acceptor
molecule" refers to any molecule which binds to a
ligand. Acceptor molecules may be biological
macromolecules such as antibodies, receptors, enzymes,
nucleic acids, or smaller molecules such as certain
20   carbohydrates, lipids, organic compounds serving as
drugs, metals, etc.
        The synthetic test compound in libraries of the
invention can potentially interact with many different
acceptor molecules. By identifying the particular
25   ligand species to which a specific acceptor molecule
binds, it becomes possible to physically isolate the
ligand species of interest.
        Because only a small number of solid support
beads will be removed during each
30   screening/detection/isolation step, the majority of
the beads will remain in the bead pool. Therefore,
the library can be reused multiple times. If
different color or identification schemes are used for
different acceptor molecules (e.g., with fluorescent
35   reporting groups such as fluorescein (green), Texas

Red (Red), DAPI (blue) and BODIPI tagged on the
acceptors), and with suitable excitation filters in
the fluorescence microscope or the fluorescence
detector, different acceptors (receptors) can be added
5   to a library and evaluated simultaneously to
facilitate rapid screening for specific targets.
These strategies not only reduce cost, but also
increase the number of acceptor molecules that can be
screened.

10   In the method of the invention, an acceptor
molecule of interest is introduced to the library
where it will recognize and bind to one or more ligand
species within the library. Each ligand species to
which the acceptor molecule binds will be found on a
15   single solid phase support so that the support, and
thus the ligand, can be readily identified and
isolated.

        The desired ligand can be isolated by any
conventional means known to those of ordinary skill in
20   the art and the invention is not limited by the method
of isolation. For example and not by way of
limitation, it is possible to physically isolate a
solid-support-bead ligand combination that exhibits
the strongest physico-chemical interaction with the
25   specific acceptor molecule. In one embodiment, a
solution of specific acceptor molecules is added to a
library which contains 10^5 to 10^7 solid phase support
beads. The acceptor molecule is incubated with the
beads for a time sufficient to allow binding to occur.
30   Thereafter, the complex of the acceptor molecule and
the ligand bound to the support bead is isolated.
More specific embodiments are set forth in the
following methods, which describe the use of a
monoclonal antibody, as a soluble acceptor molecule to
35   bind a ligand which is a peptide. It will be clear

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that these methods are readily adaptable to detect
binding of any acceptor molecule.

In addition to using soluble acceptor molecules,
in another embodiment, it is possible to detect
5 ligands that bind to cell surface receptors using
intact cells. The use of intact cells is preferred
for use with receptors that are multi-subunit or
labile or with receptors that require the lipid domain
of the cell membrane to be functional. The cells used
10 in this technique may be either live or fixed cells.
The cells will be incubated with the library and will
bind to certain peptides in the library to form a
"rosette" between the target cells and the relevant
bead-peptide. The rosette can thereafter be isolated
15 by differential centrifugation or removed physically
under a dissecting microscope.

Alternatively, one may screen the library using a
panning procedure with cell lines such as (i) a
"parental" cell line where the receptor of interest is
20 absent on its cell surface, and (ii) a receptor-
positive cell line, e.g., a cell line which is derived
by transfecting the parental line with the gene coding
for the receptor of interest. It is then possible to
screen the library by the following strategy:
25 (i) first depleting the library of its non-specific
beads that will bind to the cells lacking the receptor
by introducing a monolayer of parental cell line by
the standard "panning technique" to leave receptor-
specific non-binding beads, or irrelevant non-binding
30 beads (ii) removing the non-binding beads which will
include both receptor-specific or irrelevant beads and
loading them on a monolayer of receptor positive cell
line in which the receptor-specific bead will bind to
the receptor positive cell line, (iii) removing the
35 remaining irrelevant non-binding beads by gentle

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washing and decanting, and (iv) removing the receptor-
specific bead(s) with a micromanipulator, such as a
micropipette.

     As an alternative to whole cell assays for
5 membrane bound receptors or receptors that require the
lipid domain of the cell membrane to be functional,
the receptor molecules can be reconstituted into
liposomes where reporting group or enzyme can be
attached.

10   The foregoing examples refer to synthetic test
compound, and any of the compounds described in
Sections, supra, may be used in the practice of the
instant invention. Thus, an acceptor molecule may
bind to one of a variety of polyamides, polyurethanes,
15 polyesters, polyfunctionalized structure capable of
acting as a scaffolding, etc.

     In one embodiment, the acceptor molecule may be
directly labeled. In another embodiment, a labeled
secondary reagent may be used to detect binding of an
20 acceptor molecule to a solid phase support particle
containing a ligand of interest. Binding may be
detected by in situ formation of a chromophore by an
enzyme label. Suitable enzymes include, but are not
limited to, alkaline phosphatase and horseradish
25 peroxidase. In a further embodiment, a two color
assay, using two chromogenic substrates with two
enzyme labels on different acceptor molecules of
interest, may be used. Cross-reactive and singly-
reactive ligands may be identified with a two-color
30 assay.

     Other labels for use in the invention include
colored latex beads, magnetic beads, fluorescent
labels (e.g., fluoresceine isothiocyanate (FITC),
phycoerythrin (PE), Texas red (TR), rhodamine, free or
35 chelated lanthanide series salts, especially Eu^{3+}, to

name a few fluorophores), chemiluminescent molecules, radio-isotopes, or magnetic resonance imaging labels. Two color assays may be performed with two or more colored latex beads, or fluorophores that emit at

5   different wavelengths. Labeled beads may be isolated manually or by mechanical means. Mechanical means include fluorescence activated sorting, i.e., analogous to FACS, and micromanipulator removal means.

In specific examples, _infra_, enzyme-chromogen

10   labels and fluorescent (FITC) labels are used.

Reactive beads may be isolated on the basis of intensity of label, e.g., color intensity, fluorescence intensity, magnetic strength, or radioactivity, to mention a few criteria. The most

15   intensely labeled beads may be selected and the ligand attached to the bead may be structurally characterized directly e.g., by Edman sequencing or by mass spectral analysis if applicable, or indirectly by sequencing the coding peptide corresponding to the ligand of

20   interest. In another embodiment, a random selection of beads with a label intensity above an arbitrary cut-off may be selected and subjected to structural analysis. One can potentially use modern image analysis microscopy to quantitate the color intensity,

25   and hence precisely define the relative affinity of the ligand to the acceptor molecule prior to the structure analysis of the bead ligand. Similarly, quantitative immunofluorescence microscopy can be applied if the acceptor is tagged with a fluorescent

30   label. In yet another embodiment, beads demonstrating a certain label intensity are selected for compositional analysis, e.g., amino acid composition analysis in the case of peptide ligands. A refinement library comprising a restricted set of amino acid

35

subunits identified as important from the amino acid
analysis may then be prepared and screened.

In another embodiment, the ligand(s) with the
greatest binding affinity may be identified by

5 progressively diluting the acceptor molecule of
interest until binding to only a few solid phase
support beads of the library is detected.
Alternatively, stringency of the binding with the
acceptor molecule, may be increased. One of ordinary

10 skill would understand that stringency of binding may
be increased by (i) increasing solution ionic
strength; (ii) increasing the concentration of
denaturing compounds such as urea; (iii) increasing or
decreasing assay solution pH; (iii) use of a

15 monovalent acceptor molecule; (iv) inclusion of a
defined concentration of known competitor into the
reaction mixture; and (v) lowering the acceptor
concentration. Other means of changing solution
components to change binding interactions are well

20 known in the art.

In another embodiment, ligands that demonstrate
low affinity binding may be of interest. These may be
selected by first removing all high affinity ligands
and then detecting binding under low stringency or

25 less dilute conditions.

In a preferred embodiment, a dual label assay may
be used. The first label may be used to detect non-
specific binding of an acceptor molecule of interest
to beads in the presence of soluble ligand. Labelled

30 beads are then removed from the library, and the
soluble ligand is removed. Then specific binding
acceptor molecule to the remaining beads is detected.
Ligands on such beads may be expected to bind the
acceptor molecule at the same binding site as the

35 ligand of interest, and thus to mimic the ligand of

interest. The dual label assay provides the advantage
that the acceptor molecule of interest need not be
purified since the first step of the assay allows
removal of non-specific positive reacting beads. In a
5   preferred embodiment, fluorescent-labeled acceptor
molecules may be used as a probe to screen a synthetic
test library, e.g., using FACS.

5.6.2 BIOACTIVITY ASSAYS

10   The instant invention further provides assays for
biological activity of a ligand-candidate from a
library treated so as to remove any toxic molecules
remaining from synthesis, e.g., by neutralization and
extensive washing with solvent, sterile water and
15 culture medium. The biological activities that may be
assayed include toxicity and killing, stimulation and
growth promotion, signal transduction, biochemical and
biophysical changes, and physiological change.

In a preferred embodiment, the synthetic test
20 compound of the library are selectively cleavable from
the solid-phase support, also referred to herein as
"bead". Preferably, the synthetic test compound are
attached to the separate phase support via multiple
cleavable linkers to allow for more than one release
25 and screening assay. In one embodiment, beads are
prepared such that only a fraction of synthetic test
compound are selectively cleavable. Selectively
cleavable ligand-candidates, linkers and beads are
discussed in Section 5.3.2, supra. A library is
30 treated with a cleaving agent such that cleavage of a
fraction of synthetic test compound occurs. Examples
of cleaving agents include, but are not limited to, UV
light, acid, base, enzyme, or catalyst. In one
embodiment, the library is treated so that 10-90% of
35 the synthetic test compound are released. In a more

preferred embodiment, 25-50% of the synthetic test
compound are released. Where all synthetic test
compound molecules are cleavable, non-quantitative
cleavage can be effected by limiting the cleaving

5   agent. In one aspect, exposure time and intensity of
UV light is limited. In another embodiment, the
concentration of reagent is limited. After treatment
to effect cleavage, the library may be further
treated, e.g., by neutralization, to make it

10 biologically compatible with the desired assay. In
practice, one of ordinary skill would be able to
readily determine appropriate cleavage conditions for
partial cleavage when all synthetic test compound
molecules of the library are attached to solid phase

15 by cleavable linkers or bonds. One of ordinary skill
would further understand that the relative
concentration of released synthetic test compound can
be affected by varying the cleavage conditions.

       Since the beads of the library are immobilized, a

20 concentration gradient of a particular ligand-
candidate will form. High concentrations of synthetic
test compound will be found in proximity of the bead
from which it was released. Thus, evidence of
biological activity of interest, in proximity to a

25 bead, will allow identification and isolation of the
bead, and structural characterization by sequencing
the coding molecule corresponding to the synthetic
test compound or other technique. Identification of
the synthetic test compound is possible because enough

30 will be left on the bead after partial cleavage for
sequencing or other characterization. In another
embodiment, the beads may be partitioned in microtiter
wells (e.g., 10 beads/well) and a fraction of ligand-
candidate released and tested for biological activity,

35 thus eliminating the potential problem of diffusion.

Different portions of synthetic test compound may be
attached to solid phase support or bead via different
cleavable linkers for sequential assays. Within these
examples, the term "bead" refers to a separate phase
5 support particle.

Biological assays with uncleaved synthetic test
compound are also envisioned. The biological activity
of whole synthetic test compound-coated beads may then
be screened. In one aspect, a library may be
10 introduced into an animal. Beads of interest may be
isolated from a specific tissue. Beads may be
isolated that were specifically absorbed after oral,
nasal, or cutaneous administration. In a preferred
embodiment, such beads are magnetic, or have some
15 other identifying feature, and thus are readily
isolated from the tissue. In another embodiment,
immobilized ligand itself may elicit biochemical
changes with appropriate surface receptors.

It will further be understood by one of ordinary
20 skill in the art that any cell that may be maintained
in tissue culture, either for a short or long term,
may be used in a biological assay. The term "cell" as
used here is intended to include prokaryotic (e.g.,
bacterial) and eukaryotic cells, yeast, mold, and
25 fungi. Primary cells or lines maintained in culture
may be used. Furthermore, applicants envision that
biological assays on viruses may be performed by
infecting or transforming cells with virus. For
example, and not by way of limitation, the ability of
30 a ligand to inhibit lysogenic activity of lambda
bacteriophage may be assayed by identifying
transfected E. coli colonies that do not form clear
plaques when infected.

Methods of the present invention for assaying
35 activity of a synthetic test compound molecule of a

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library are not limited to the foregoing examples;
applicants envision that any assay system may be
modified to incorporate the presently disclosed
invention. Applicants envision that such are within
5   the scope of their invention.

5.6.3 ENZYME MIMICS/ENZYME INHIBITORS

The present invention further comprises libraries
that are capable of catalyzing reactions, i.e., enzyme
10 libraries; libraries of molecules that serve as co-
enzymes; and libraries of molecules that can inhibit
enzyme reactions. Thus, the invention also provides
methods to be used to assay for enzyme or co-enzyme
activity, or for inhibition of enzyme activity.

15   Enzyme activity may be observed by formation of a
detectable reaction product. In a particular
embodiment, an enzyme from an enzyme library catalyzes
the reaction catalyzed by alkaline phosphatase, e.g.,
hydrolysis of 5-bromo-4-chloro-3-indoyl phosphate
20 (BCIP) and forms a blue, insoluble reaction product on
the solid phase support (see Example 13, infra).

In another embodiment, a zone of observable
product, e.g., color or fluorescence, may be formed in
a semi-solid matrix. A library is layered in a semi-
25 solid matrix, e.g., agarose gel, and a chromogenic or
other indicator substrate is added. Where an enzyme-
bead complex from an enzyme library shows the
desirable enzyme activity, a zone of product will
form. For example, and not by way of limitation, a
30 molecule from a library which is an analog of
horseradish peroxidase may be identified by adding a
solution of aminoantipyrene (0.25 mg/ml; Kodak),
phenol (8 mg/ml) and H_2O_2 (0.005%) in 0.1 M phosphate
buffer, pH 7.0. Beads with enzyme activity will form
35 a purple zone of color. In another embodiment, beads

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with protease activity may be identified by addition
of the well known colorimetric protease substrates.

Co-enzyme activity may be observed by assaying
for the enzyme activity mediated by a co-enzyme, where
5   the natural or common co-enzyme is absent.

Enzyme inhibitory activity can be detected with a
partially-released synthetic test compound. In one
example, and not by way of limitation, a library is
layered in a semi-solid matrix that contains an
10 enzyme. The library is treated to partially release
ligand-candidate molecules. Where the molecule
inhibits the enzyme activity, a zone lacking product
may be identified. In one embodiment, the enzyme
substrate is chromogenic, and a colored product is
15 formed. Thus, presence of an enzyme inhibitor would
yield a zone of no color. In another embodiment,
inhibition of proteolysis of hemoglobin or an
indicator enzyme such as alkaline phosphatase may be
detected by the presence of an opaque zone in the
20 semi-solid matrix. This is because presence of
proteolysis inhibitor will prevent degradation of the
hemoglobin or indicator enzyme.

It will be well known to one of ordinary skill
that a synthetic test compound molecule that
25 demonstrates enzyme activity, co-enzyme activity, or
that inhibits enzyme activity, may be a peptide, a
peptide mimetic, one of a variety of polymers or any
of the compounds described in Section 5. Of
particular interest are the constrained polymers,
30 including but not limited to cyclic, bicyclic or
tricyclic structures, or constrained structures with
certain scaffolding, which can create an unique
catalytic binding pocket or surface.

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5.6.4   TOPOLOGICAL SEGREGATION

The invention further encompasses a method of segregating the coding molecule in the interior of the solid support and the test compound on the exterior,

5   accessible to a macromolecular acceptor molecule of interest. The method encompasses the steps of synthesizing a linker, which in the preferred embodiment is a peptide. The linker contains a sequence which can be hydrolyzed by a conveniently

10   available enzyme such as chymotrypsin or other endopeptidase. In one embodiment the enzyme is chymotrypsin and the linker contains a phenylalanine. After the linker is synthesized, the N^α amino function is left protected and the support is exposed to the

15   endopeptidase. The endopeptidase acts only on linkers that would be accessible to other macromolecules, such as acceptors.

After the enzymatic hydrolysis of the peptide linker, the test compound and the coding compounds can

20   be synthesized using any orthogonal protecting groups.

5.7 METHODS OF CHARACTERIZING A SYNTHETIC TEST
     COMPOUND FROM A LIBRARY

Once a support containing a ligand of interest is selected according to any one of the methods of

25   Section 5.6, supra, the present invention provides a means of determining the structure of the ligand.

There are two general approaches to determining the structure of a test compound: the structure of the polymer may be directly analyzed by conventional

30   techniques, e.g., Edman degradation or mass spectrometry; alternatively, a second molecule or group of molecules can be synthesized during the construction of the library such that the structure(s) of the second molecular species unambiguously

35   indicates (encodes) the structure of the test compound

attached to the same support. By this second
technique, the structure of polymers that are not
themselves amenable to sequencing can be readily
determined.

5   Yet another embodiment of the present invention
encompasses a third coding technique, termed
"fractional coding," which differs from the previous
embodiments in that there is not a distinct coding
molecule different from the test compound. Functional

10 coding is used when specific subunits of the test
compound are not resolvable in conventional analysis,
e.g., the D and L stereo isomers of an amino acid.
Fractional coding provides a method whereby the
subunits can be distinguished by mixing a small amount

15 of a different subunit one, not otherwise utilized in
the construction of the library, at the time the
library is synthesized. Thus, fractional coding
creates a minor, detectable degree of heterogeneity of
the test compound of the support when one of the two

20 indistinguishable subunits is used. For the purposes
of the present invention such a degree of
heterogeneity, typically about 5%, is compatible with
the teaching of the application that there be only one
species of test compound on each support.

25

5.7.1   CHARACTERIZATION BY MEANS OF SINGLE
AND MULTIPLE SEQUENTIAL CODES

In a preferred embodiment of the encoded
molecular libraries, the separate phase support
containing the synthetic test compound of interest

30 also contains a molecule, preferably a peptide, whose
sequence encodes the structure of the ligand, e.g.,
determination of the sequence of the coding peptide
reveals the identity of the ligand. A preferred

35 method of determining the peptide sequencing is Edman
degradation. A particularly preferred method employs

the Applied Biosystems 477A Protein Sequencer. The amino acid sequence of peptides can also be determined either by fast atom bombardment mass spectroscopy (FAB-MS) or using other analytical techniques.

5　　　The coding peptides can be sequenced either attached to or cleaved from the solid support. To cleave the peptides, the isolated beads are treated with traditional cleaving agents known to those of skill in this art to separate peptides from solid

10　phase supports. The choice of cleaving agent selected will depend on the solid phase support employed.

　　　Alternatively, in another embodiment within the scope of the invention, it is possible to isolate a single solid phase support particle, such as a bead,

15　with its coding peptide sequence attached and introduce the bead to a sequencer for peptide sequencing without previously cleaving the coding peptide from the bead. It is estimated that a single 100 μm diameter resin bead with 0.5 mEq/gram of

20　functionalizable sites contains approximately 100 pmole of peptide if one half of the sites are used to link coding peptides. For a similar degree of substitution with coding peptides, a single 250 μm diameter PAM resin bead with 0.5 mEq/gram resin of

25　functionalizable sites contains approximately 1500 pmole of coding peptide. With a state of the art peptide sequencer, only 5-10 pmole is required for adequate sequencing. Therefore, for a standard PAM resin a single bead of 100 μm in diameter can be

30　loaded to contain more than an adequate amount of coding peptide for sequencing.

　　　In addition to Edman sequencing, fast ion bombardment mass spectrometry is a very powerful analytical tool and can often be used effectively to

35　analyze the structures of peptides and of a variety of

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other molecules. Electrospray-high performance mass
spectrometry can also be very useful in structural
analysis. Preferably, mass spectrometry to determine
the structure of a coding molecule is performed as

5   described in U.S. Patent Application Serial No.
07/939,811, filed September 3, 1992.

    Those skilled in the art will appreciate that at
times the number of species of subunits at any
position of the test compound is larger than the

10 number of monomers used to construct the coding
polymer. For example, a coding peptide can be
constructed with a limited set of amino acids that are
readily distinguished after Edman degradation. Under
these circumstances the coding molecule can be

15 constructed by introducing a mixture of amino acids at
a given position. For example a singlet/doublet code,
i.e., having one or two coding moieties per position
of the test compound, in which the coding polypeptide
contains only 8 amino acids can encode up to 36

20 subunits; a triplet/doublet/singlet code with the same
number of moieties encodes 84 subunits per position.

    The analysis of the Edman degradation products of
such coding peptides will reveal either one or two, or
one, two or three animo acids at each position of the

25 coding sequence.

        5.7.2   CHARACTERIZATION BY MEANS OF A
                 NON-SEQUENTIAL CODE

        An alternative preferred embodiment, termed non-
30 seqential coding allows for the "reading" of the
coding molecule without a determination of the
sequence of its subunits. Sequential codes are
inherently laborious to decode. Sequencing of a
molecule requires reiterated degradative steps. By

35 contrast analysis of the composition of a polymeric
molecule can be performed by a single degradation and

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a single analysis of the resultant subunits or their derivatives.

Further, the most time consuming of the steps required to sequence a peptide coding molecule is the
5   chromatographic analysis of each of the cleaved phenylthiohydantoins. Even though all the information resides in the unique retention time of the single eluted peak, a separate chromatography step must be performed on the products of each step-wise
10 degradation. Thus, most of the time of gradient analysis is "wasted" waiting for the appearance of the single peak corresponding to the residue at that position. The process is clearly not as efficient as possible. If instead of sequential Edman degradation
15 followed by HPLC analysis, it were possible to simultaneously cleave all the coding subunits, distinguish among them in a single HPLC run, and then decode the results to determine the test compound identity, the process could be greatly accelerated.
20   The reading of a non-sequential code requires only determining whether a given signal is present or not. The baseline resolution of two peaks which differ by about 0.3 minutes in retention time can be achieved using standard reversed phase HPLC analysis
25 with gradient elution. Therefore, a 45 minute gradient can discrimate among 150 compounds. A coding molecule consisting of subunits selected from a group of 150 different coding moieties is equivalent to a 150 digit binary number. Hence, 2^{150} or about 10^{45} different
30 species of test compound could be so encoded. Thus, non-sequential codes are easily adequate to encode both the sequence and the identity of the subunits of the test compounds of even the largest practical libraries.
35

A non-sequential code can be constructed as
follows. Let C000 to C099 be the elements of the set
of 100 coding moieties to be used to encode the
structure of a test compound having up to 20 residues
5 selected from up to 32 different subunits, called here
S00-S31. In this scheme the identity of the residue
at the first position is determined by the presense or
absence of coding elements C000-C004; if none are
present S00 is present at the first position of the
10 test compound, if all are present S31 is present at
position 1. Successive positions are encoded by
moieties C005-C009, C010-C014 . . . C095-C099. Those
of ordinary skill will understand that, in the
frequent case wherein libraries considerably smaller
15 than the maximum coding capacity of a 100 digit code
are required; the fidelity of the code may be
increased by either reducing the size of the set of
coding moleules, i.e., increasing the interval between
moieties in the chromatographic analysis, or by the
20 use of redundant coding, e.g., a "parity" moiety may
be introduced into the code for each encoded position.

Most frequently, between 4 and 8 coding moieties,
corresponding to between 16 subunits and 128 subunits
plus a parity moiety, will be required to encode each
25 position in the test compound.

The coding moieties need to be arranged in the
coding structure to allow their simultaneous cleavage
and analysis. One obvious possibility is total
hydrolysis, followed by selective modification and
30 analysis of the mixture. In this case the structure of
the coding compound is not important. Coding moieties
can be connected to one another, or attached to
separate branches of a branched structure, or any
combination so long as the bond to each moiety is
35 hydrolyzable. This approach, however, might be

compromised by the presence of hydrolytic products
from the test compound. Therefore, the use of the very
selective degradation method designed by Edman, 1950,
ACTA CHEM SCAND, 4:283-293; Edman, et al., 1967, EURO J
5   BIOCHEM, 1:80-91 seemed the optimal choice.

Edman degradation selectively cleaves the N-
terminal amino acid from the peptide chain. If
reasonable number of amino acids and amino acid
derivatives fulfilling the chromatographic
10 requirements defined above could be identified, and
they were synthesized as a coding structure in an
arrangement allowing their simultaneous cleavage, it
would be possible to analyze the composition of a
nonpeptidic structure in just one cycle of Edman
15 degradation and HPLC analysis.

The retention time of amino acid
phenylthiohydantoins on reversed phase follows the
lipophilicity of the side chain of the amino acid.
Thus, to design a set of amino acid derivatives with
20 the appropriate retention times it is only necessary
to design the side chain of each with appropriate
differences in lipophilicity. One simple way to
achieve the appropriate differences is to substitute
the functional group of the side chains of
25 trifunctional amino acids by appropriate substituents.
Consequently, we explored the effect of acylating the
side chain amino group of diamino carboxylic acids -
diaminopropionic acid, diaminobutyric acid, ornithine
and lysine. Alternative sets of coding moieties such
30 as derivative dicarboxyamino acids and SH - containing
amino acids, will be readily apparent to those skilled
in the art. The above described moiety is preferred
only in that it can be conveniently synthesized by
those having ready access to solid phase peptide
35 synthesis.

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One embodiment to achieve the simultaneous cleavage of coding moieties provides that every coding moiety is an α-amino acid, attached as an N-terminal amino acid with its amino group free. The backbone of

5   such a coding structure can be constructed from diamino carboxylic acids (Daa) dicarboxy amino acids or other trifunctional subunits. The amino groups of these amino acids are acylated by the N-protected amino acids used for the coding. Acylation is

10   performed using a mixture of the moieties defined as the code for the given subunit and its position in the test compound. As illustrated in Scheme XIC, *infra*, the positional chemistry of the reactions of the diamino carboxylic acids need not be specified. The

15   polymer and the coding moieties may be coupled to either of the amino groups of the diamino acids that form the coding molecule polymer.

In the case of the substituted diamino carboxylic acids described in Example 13, *infra*. and illustrated

20   the coupling reactivities were independent of side chain substitutions. However, should other coding subunits be used, there are two methods which can achieve equimolar incorporation of the coding moieties even though they may have significantly different

25   reactivities, e.g., alanine and isoleucine. The first method is based on compensating the differences in reactivity by using a higher concentration of the slower reacting amino acid (e.g., Eichler J. & Houghten, R., 1993, Biochemistry 32:11035; Rutter U.S.

30   Pat. No. 5,010,175). Alternatively, a subequimolar amount of the mixture can be used repetitively so that even though one moiety might react faster, sufficient coupling sites remain for the slower reacting amino acid to couple, until after sufficient repetitions of

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coupling all the reactive sites are consumed. Andrews
et al., 1994, TECHNIQUES IN PROTEIN CHEMISTRY, 5:485-492.

There are several basic strategies for the
construction of a coding molecule as described above.
Two of them are illustrated in scheme XIA. The first
is based on the use of the Alloc protecting group
(Loffet A. & Zhang H., 1993, Int. Pep. & Prot. Res.
42:346); (Stevens & Watanabe, 1950 J. Am. Chem. Soc.
72:725); (Guibe, F. & Saint M'Leux Y., 1981, Tet.
Lett. 21:3591) for building the coding structure and
the Fmoc or Fmoc-like group (Carpino & Han, 1972 J.
Org. Chem. 37:3404) for the protection of functional
groups on the test compound. In this case the Boc
group can be used as the permanent protecting group
for both the test compound synthesis and coding
synthesis. It is advantageous to use preformed coding
subunits of the general form of "block A" depicted in
scheme XIA. Alternatively, if preformed coding
subunits are not used, another level of orthogonality
is required during synthesis. This can be achieved by
using Alloc/Ddz protected diamino carboxylic acids for
building the coding backbone, since the Ddz group is
selectively cleavable by 2% trifluoroacetic acid in
dichloromethane (Birr C., et al., 1972, Liebig's Ann.
Chem. 763:162-73). However, this approach is
complicated by the need to compensate for different
coupling reactivities of the coding amino acids
attached as a mixture. A second strategy is based on
the use of a combination of Fmoc and Boc groups for
temporary orthogonal protection of functional groups
in the test compound and coding molecules, and the use
of benzoxycarbonyl (Z) or Z-like groups for permanent
protection. The coding subunit can be built during
the synthesis using Fmoc/Dde (or Fmoc/Alloc, or
Fmoc/Ddz) protected diamino carboxylic acids since the

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Dde group is cleaved by a solution of hydrazine in
dimethylformamide and is stable under conditions used
for removal of the Boc or Fmoc group (Hone, N. D.
et al., Poster P63 at 22nd Eur. Pept. Symp.,

5   Interlaken, Switzerland, September 1992).
        An alternative approach for the coding is
illustrated in Scheme XIB. In this case, a fraction
of the amino groups available for coding are acylated
by the coding mixture and the remaining amino groups

10   are reprotected by an orthogonally cleavable group
(e.g. Alloc) before the next step of randomization is
performed. In this scheme it is advantageous to couple
the coding mixture prior to the test compound subunit
since the deprotection of Alloc can be performed in

15   the recombined stage.
        A third alternative embodiment is provided by
Scheme XIC. In this scheme, the coding elements need
not be preformed with a backbone diaminocarboxylic
acid, as in Scheme XIA, nor need there be repetitive

20   blocking and deblockings as in Scheme XIB. The scheme
provides an Fmoc-protected test compound and an Alloc-
protected coding molecule, with both cooding and test
compound reactive groups permanently protected during
synthesis with Boc. The coding molecule is

25   synthesized by the sucessive steps of removing Alloc
protection of both the N^a and N^e functionalities of the
lysine; addition and coupling of a mixture of coding
elements $Boc\text{-}Caa_{11}$ and $Boc\text{-}Caa_{12}$, each in the amount of
0.5 equivalent, addition and coupling of 1.0

30   equivalent of Alloc-Lys(Alloc). Note that in this
embodiment the coding elements, i.e., the Boc-Caa's,
may couple to either the N^a and/or the N^e of any
particular lysine in the coding molecule backbone.
Nonetheless neither the stereochemistry nor

35   stoichiometry of the coupling effects the operability

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of the embodiment because for all purposes of the
invention the N^{α} and N^{ϵ} functionalities of lysine are
equivalent.

5   Yet another alternative embodiment of the
invention encompasses the reading of a non-sequential
code by mass-spectroscopy. The coding molecules are
released from the solid phase support by specific
cleavage of a linker. The molecules can be further
fragmented by electro/spray or ionic bombardment and
10   the individual coding moieties identified by their
molecular weights.

     Those skilled in the art will appreciate that the
use of mass spectroscopy to read a non-sequential code
implies that the coding moieties may be linked to the
15   coding molecules by a variety of chemical bonds other
than those susceptible to specific degradation, such
as peptide bonds.

     Schemes XIA, XIB and XIC follow:

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Fmoc — Bead

Boc

1. - Boc
2. + Boc-Caa$_{11}$ + Boc-Caa$_{12}$ (25%)
3. + Alloc-Cl

5

Fmoc — Bead

Boc-Caa$_{11}$
Boc-Caa$_{12}$

Alloc — NH

1. - Fmoc
2. + Fmoc-Block(Boc)

10

Fmoc-Block(Boc) — Bead

Boc-Caa$_{11}$
Boc-Caa$_{12}$

15

Alloc — NH

Boc

Fmoc — Screening structure — Bead

Boc-Caa$_{11}$
Boc-Caa$_{12}$
Boc-Caa$_{21}$
Boc-Caa$_{22}$
Boc-Caa$_{31}$
Boc-Caa$_{32}$
Boc-Caa$_{41}$
Boc-Caa$_{42}$

NH

20

25

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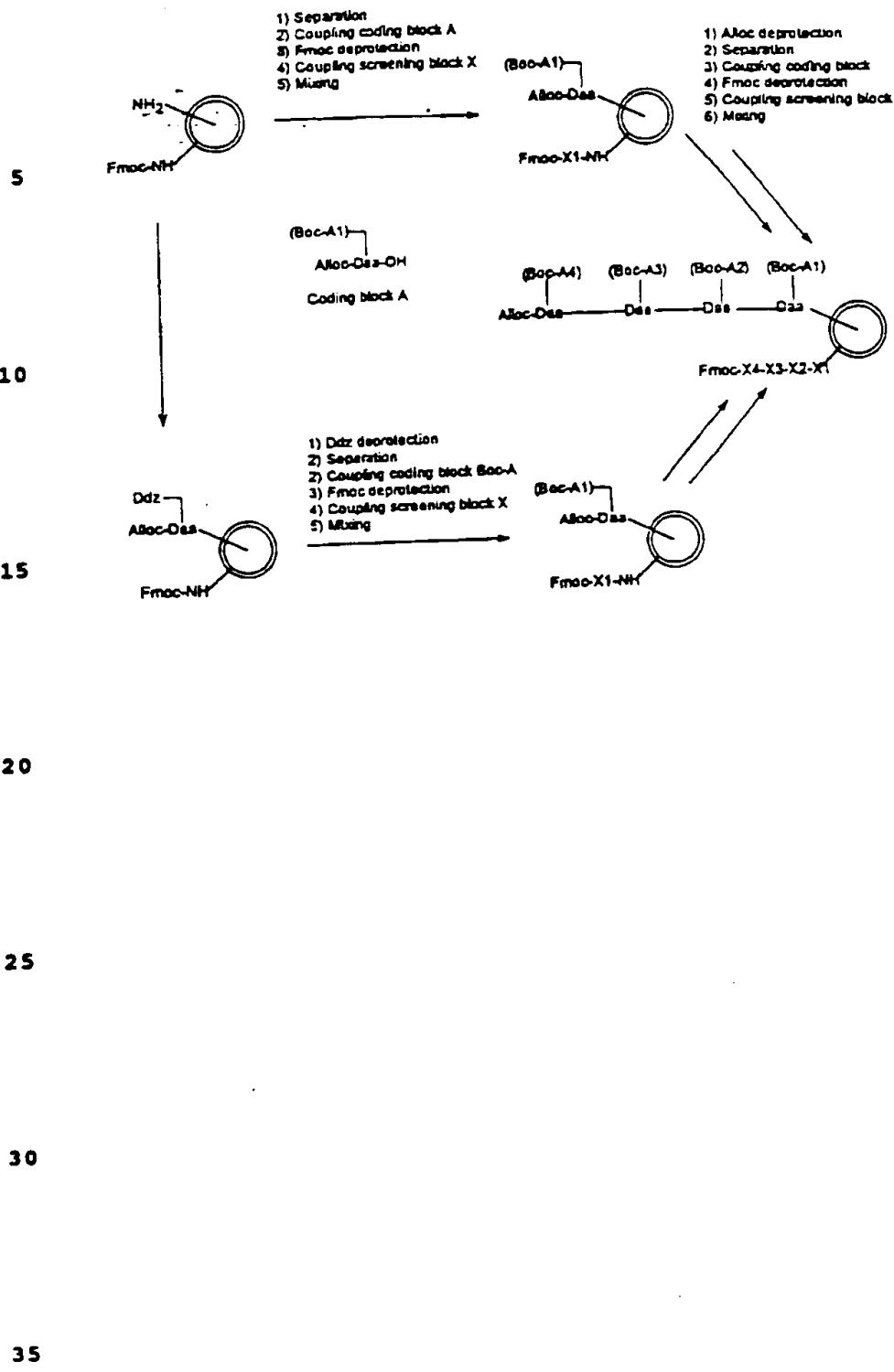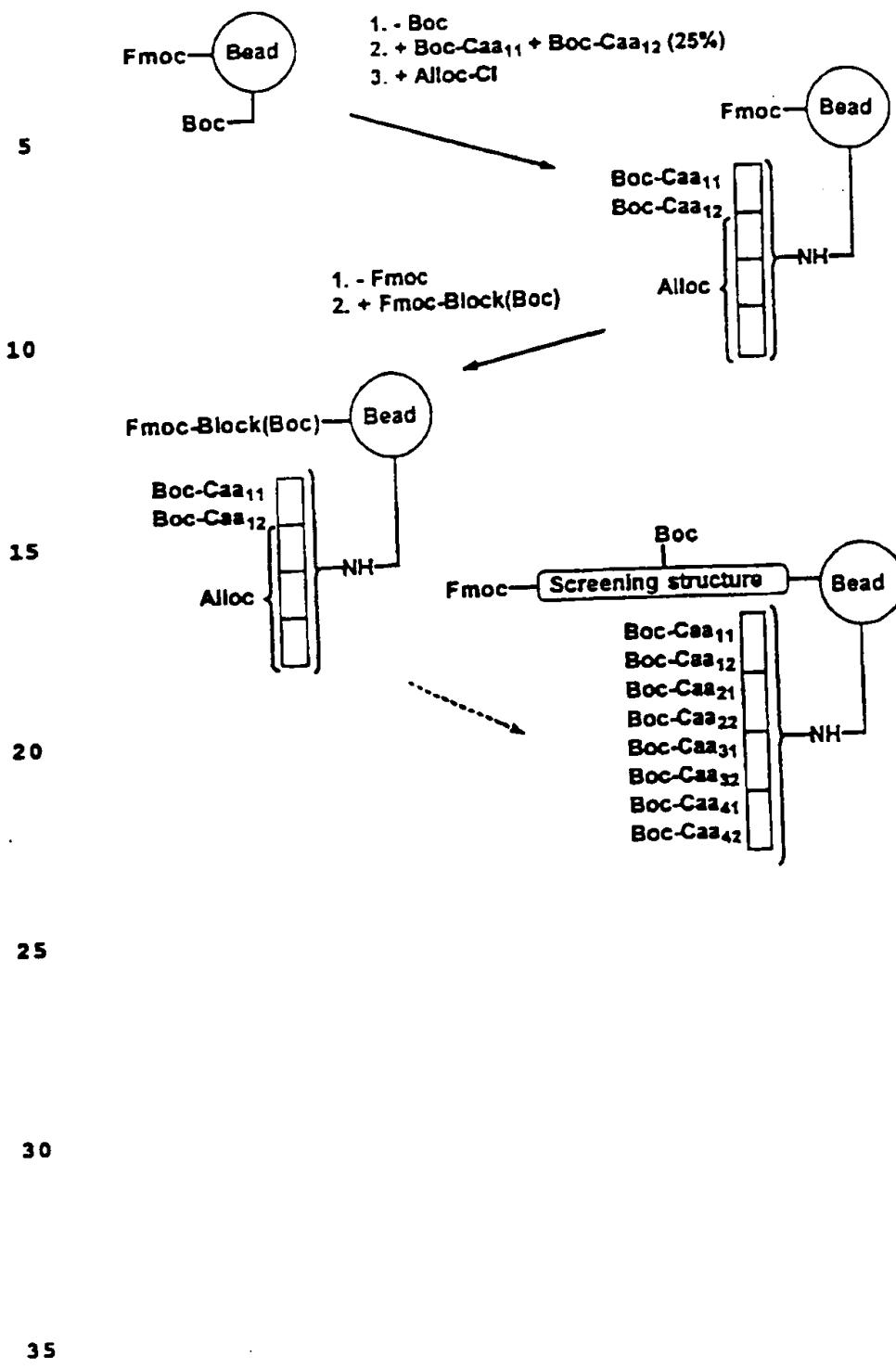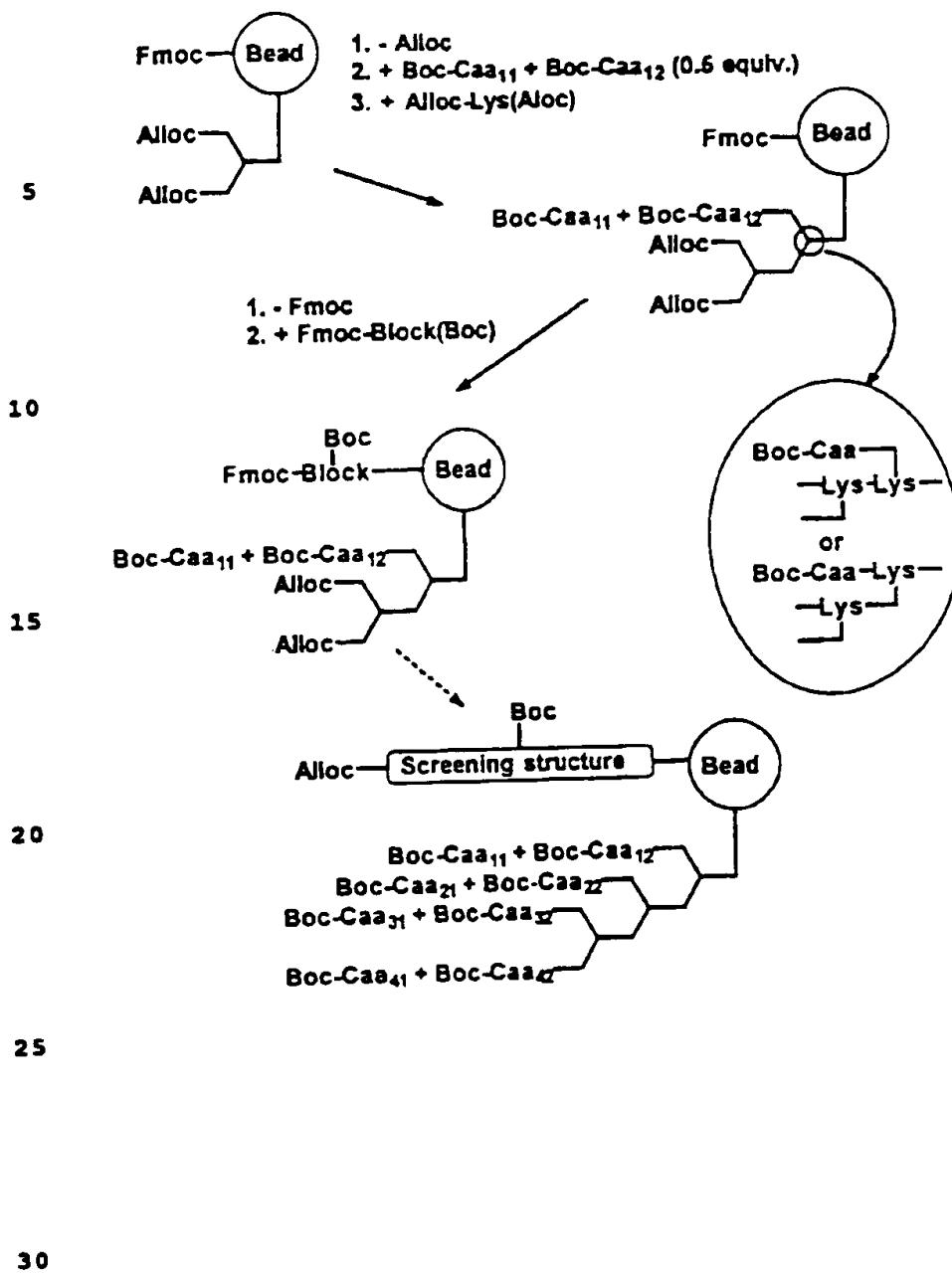
5.7.3 NON-CODED LIBRARIES

For the analysis of the structure of ligands selected from libraries which do not contain coding molecules, the technique used to analyze the structure of coding peptides (described above) may be used when applicable. Alternatively, mass spectrometry particularly using techniques described in U.S. Application Serial No. 07/939,811 filed September 3, 1992, or other analytical techniques (thin layer chromatography, HPLC, NMR, IR, elemental analysis, and the like) can be used to determine the structure of a synthetic test compound selected according to the present invention.

5.8 THERAPEUTIC AND DIAGNOSTIC AGENTS FROM LIBRARIES OF Synthetic test compound

Once the structure of a selected ligand is determined, a large amount of the compound may be synthesized chemically or biologically for confirmation of the results of the structural and screening experiments and other studies. Once a molecular structure of interest has been identified through library screening and structural analysis of active ligands, the present invention provides molecules that comprise the molecular structure for use in treatment or diagnosis of disease. The molecule identified through screening alone may provide a diagnostic or therapeutic agent, or may be incorporated into a larger molecule. A molecule comprising a structure with biological or binding activity may be termed an "effector molecule." The invention further provides libraries for use in various applications. The "effector" function of said effector molecule may be any of the functions described herein or known in the art.

The method described herein not only provides a new tool to search for specific ligands of potential

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diagnostic or therapeutic value, but also provides important information on a series of ligands of potentially vastly different structure which nonetheless are able to interact with the same

5 acceptor molecule. Integrating such information with molecular modeling and modern computational techniques is likely to provide new fundamental understanding of ligand-receptor interactions.

The therapeutic agents of the invention comprise

10 effector molecules that will bind to the biologically active site of cytokines, growth factors, or hormonal agents and thereby enhance or neutralize their action, and that will block or enhance transcription and/or translation.

15 The therapeutic agents of the invention include, for example, effector molecules that bind to a receptor of pharmacologic interest such as growth factor receptors, neurotransmitter receptors, or hormone receptors. These effector molecules can be

20 used as either agonists or antagonists of the action of the natural receptor ligand.

Another application of effector molecules that bind to receptors would be to use the binding to block the attachment of viruses or microbes that gain access

25 to a cell by attaching to a normal cellular receptor and being internalized. Examples of this phenomenon include the binding of the human immunodeficiency virus to the CD4 receptor, and of the herpes simplex virus to the fibroblast growth factor receptor.

30 Effector molecules that occupy the receptor could be used as pharmacologic agents to block viral infection of target cells. Parasite invasion of cells could be similarly inhibited, after suitable effector molecules were identified according to this invention.

35

In another embodiment, an effector molecule comprising a structure that binds to an acceptor molecule of interest may be used to target a drug or toxin. In a preferred embodiment, the acceptor

5   molecule of interest is a receptor or antigen found on the surface of a tumor cell, animal parasite, or microbe, e.g., bacterium, virus, unicellular parasite, unicellular pathogen, fungus or mold. In another embodiment, the targeted entity is an intracellular

10 receptor.

In addition, it is possible that a few of the millions of synthetic test compound molecules in the pool may provide structures that have biological activity. One may isolate molecules that possess

15 antitumor, anti-animal parasite, or antimicrobial, e.g., anti-weed, anti-plant parasite, antifungal, antibacterial, anti-unicellular parasite, anti-unicellular pathogen, or antiviral activities. In addition some of these ligands may act as agonists or

20 antagonists of growth factors, e.g., erythropoietin, epidermal growth factor, fibroblast growth factor, tumor growth factors, to name but a few, as well as hormones, neurotransmitters, agonists for the receptors, immunomodulators, or other regulatory

25 molecules.

The therapeutic agents of the invention also include effector molecules comprising a structure that has a high affinity for drugs, e.g., digoxin, benzodiazepam, heroine, cocaine, or theophylline.

30 Such molecules can be used as an antidote for overdoses of such drugs. Similarly, therapeutic agents include effector molecules that bind to small molecules or metal ions, including heavy metals. Molecules with high affinity for bilirubin will be

35

useful in treatment of neonates with
hyperbilirubinemea.

In general, the present invention envisions
providing methods to identify molecules for therapy of

5   diseases or illnesses such as are listed in the
Product Category Index of The Physicians Desk
Reference (PDR, 1993, 47th Edition, Medical Economics
Data: Oradell, NJ, pp. 201-202). For example, an
effector molecule with anti-cancer, antiparasite,

10 anticoagulant, anticoagulant antagonist, antidiabetic
agent, anticonvulsant, antidepressant, antidiarrheal,
antidote, antigonadotropin, antihistamine,
antihypertensive, antiinflammatory, antinauseant,
antimigraine, antiparkinsonism, antiplatelet,

15 antipruritic, antipsychotic, antipyretic, antitoxin
(e.g., antivenin), bronchial dilator, vasodilator,
chelating agent, contraceptive, muscle relaxant,
antiglaucomatous agent, or sedative activity may be
identified.

20   The therapeutic agents of the invention may also
contain appropriate pharmaceutically acceptable
carriers, diluents and adjuvants. Such pharmaceutical
carriers can be sterile liquids, such as water and
oils, including those of petroleum, animal, vegetable

25 or synthetic origin, such as peanut oil, soybean oil,
mineral oil, sesame oil and the like. Water is a
preferred carrier when the pharmaceutical composition
is administered intravenously. Saline solutions and
aqueous dextrose and glycerol solutions can also be

30 employed as liquid carriers, particularly for
injectable solutions. Suitable pharmaceutical
excipients include starch, glucose, lactose, sucrose,
gelatin, malt, rice, flour, chalk, silica gel,
magnesium carbonate, magnesium stearate, sodium

35 stearate, glycerol monostearate, talc, sodium

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chloride, dried skim milk, glycerol, propylene,
glycol, water, ethanol and the like. These
compositions can take the form of solutions,
suspensions, tablets, pills, capsules, powders,
5 sustained-release formulations and the like. Suitable
pharmaceutical carriers are described in "Remington's
Pharmaceutical Sciences" by E.W. Martin. Such
compositions will contain an effective therapeutic
amount of the active compound together with a suitable
10 amount of carrier so as to provide the form for proper
administration to the patient. While intravenous
injection is a very effective form of administration,
other modes can be employed, such as by injection, or
by oral, nasal or parenteral administration.
15   A molecule comprising a structure determined
according to this invention may also be used to form
diagnostic agents. The diagnostic agent may also be a
molecule comprising one or more structures identified
as a result of library screening, e.g., more than one
20 polyamide sequence or polyalkane sequence. In
addition, the diagnostic agent may contain any of the
carriers described above for therapeutic agents.
        As used herein, "diagnostic agent" refers to an
agent that can be used for the detection of conditions
25 such as, but not limited to, cancer such as T or B
cell lymphoma, and infectious diseases as set forth
above. Detection is used in its broadest sense to
encompass indication of existence of condition,
location of body part involved in condition, or
30 indication of severity of condition. For example, a
peptide-horseradish immunoperoxidase complex or
related immunohistochemical agent could be used to
detect and quantitate specific receptor or antibody
molecules in tissues, serum or body fluids.
35 Diagnostic agents may be suitable for use _in vitro_ or

in <u>vivo</u>. Particularly, the present invention will provide useful diagnostic reagents for use in immunoassays, Southern or Northern hybridization, and <u>in situ</u> assays.

5   In addition, the diagnostic agent may contain one or more markers such as, but not limited to, radioisotope, fluorescent tags, paramagnetic substances, or other image enhancing agents. Those of ordinary skill in the art would be familiar with the
10   range of markers and methods to incorporate them into the agent to form diagnostic agents.

The therapeutic agents and diagnostic agents of the instant invention may be used for the treatment and/or diagnosis of animals, and more preferably,
15   mammals including humans, dogs, cats, horses, cows, pigs, guinea pigs, mice and rats. Therapeutic or diagnostic agents may also be used to treat and/or diagnose plant diseases.

The diseases and conditions amenable to therapy
20   or diagnosis with molecules discovered according to the present invention are as varied and wide-ranging as the permutations of structures in a library.

In another embodiment, low affinity-binding beads may be selected, and a limited library prepared based
25   on the structure of the ligands on the beads. In another embodiment, a custom low affinity or high affinity support comprising one or a few ligands identified from the millions of synthetic test compound provided by the invention may be used in
30   chromatographic separations.

The invention will be further clarified by the following examples, which are intended to be purely exemplary of the invention.

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6.   EXAMPLE: A MODEL ENCODED LIBRARY

       The present Example demonstrates that two
molecules, a "synthetic test compound" and a "coding
molecule", can be prepared simultaneously on a single
5   resin bead.   Furthermore, a library of such compounds
can be prepared, in which each resin bead in the
library contains a single species of the "synthetic
test compound" and a single species of "coding
molecule".   In this example, they are present in a 2:1
10   molar ratio, respectively.   The sequence of the
"coding molecule" corresponds to the sequence of the
"synthetic test compound".

       For this model system, both the "synthetic test
compound" and the "coding molecule" were peptides.
15   Parallel synthesis of the test peptide and the coding
peptide proceeded with use of orthogonal blocking
groups Boc and Fmoc.

6.1   MATERIALS AND METHODS

20   Solid phase synthesis was performed manually in
polypropylene syringes as described by Krchnak and
Vagner (1990, Peptide Res. 3:102-193).   Syntheses were
performed on TentaGel (TG) (Rapp Polymere, Tubingen,
Germany, 130 or 80 μm, 0.23mmol/g) modified with SCAL
25   handle (Patek and Lebl, 1991, Tetrahedron Lett.
32:3891-3894) (safety-catch amide linker), or with an
appropriate linker.   Cleavages of Fmoc protecting
groups were carried out with 50% piperidine/DMF for
1x10min.   The Boc protecting group was cleaved with
30   30% TFA/DCM containing 3% of anisole for 20 min.   A
solution of DIEA/DCM (10%) was used for neutralization
after Boc cleavage.   A mixture of BOP/HOBt/DIEA
(1:1:2eq) in DMF was used for activation of both Nα-
Fmoc and Boc amino acids.   The completeness of each
35   condensation reaction (1.5 - 40 hrs) was checked by

ninhydrin test or by chloranil test in the cases of
coupling to secondary amino groups. The coupling
protocol included washing with DMF (6-8 times)
(followed by washing with DCM in case of Boc protected
5   amino acids) between coupling and deprotection and
between deprotection and coupling. Reduction of SCAL
linker was performed by 20% (EtO)$_2$P(S)SH in DMPU for 2
hours. Final cleavage was done by 95% TFA - 5% water
mixture.

10   Commercial-grade solvents were used without
further purification. Protected amino acids were
obtained from Bachem (Torrance, CA), Advanced ChemTech
(Louisville, KY), or Propeptide (Vert-le-Petit,
France).

15

    6.2   RESULTS
        6.2.1   SYNTHESIS OF MODEL LIBRARY AND
                  DEPROTECTION OF BOTH PROTECTING
                  GROUPS

         Boc-Lys(Fmoc)-OH was coupled as a first amino
20   acid to SCAL-TG, the Nε-Fmoc group was deprotected and
Fmoc-Lys(Fmoc)-OH was coupled to the side chain of the
first lysine. Nα- and Nε-Fmoc groups of lysine were
cleaved and the resin was divided into three parts.
Fmoc-Ala-OH, Fmoc-Phe-OH and Fmoc-Val-OH,
25   respectively, were coupled to each portion of the
resin. Corresponding Boc amino acids (Gly, Tyr and
Leu - Boc-Tyr-OH was used with unprotected hydroxyl
group) were coupled in the next step to the α-amino
group of lysine after Boc deprotection, while the
30   "Fmoc branch" was left protected. After the
completion of Boc amino acids condensations, all three
portions of the resin were combined and the "Fmoc
branch" was deprotected. The following randomization
was performed exactly the same way as the first one
35   after the splitting of the resin into the three equal

- 95 -

portions. After randomization of three positions
(coupling of three different amino acids in each
position), the resin was divided into separate
portions for subsequent analysis.

5   Two completely deprotected beads selected at
random were separately submitted for sequence
analyses. Correct "complementary" amino acids were
found in all three cycles in the expected ratio of
2:1. Results (values in pmoles): 1st bead: 1st cycle:

10 V 251, L 146, 2nd cycle: V 244, L 147, 3rd cycle: V
245, L 119; 2nd bead: 1st cycle: A 102, G 39, 2nd
cycle: V 121, L 59, 3rd cycle: F 125, Y 50.

        Part of the resin (about 100 mg) was treated with
20% diethyldithiophosphate in DMPU (2 x 1h shaking) to

15 reduce the SCAL handle. The mixture of peptides was
cleaved from the reduced SCAL with TFA/H_2O (95:5) for
1h. The cleavage mixture was concentrated in vacuo
and precipitated with Et_2O. The precipitate was
collected by centrifugation and dried. The mixture of

20 peptides was dissolved in 0.1% TFA/H_2O and analyzed by
HPLC. A slow gradient of 0-50% acetonitrile and 0.1%
TFA over 200 min eluted the expected 27 peaks.
Several additional minor peaks were identified, the
formation of which was attributed to the use of side

25 chain unprotected tyrosine during the synthesis.
Since the danger of elimination (or at least
decreasing the content) of some sequences by ether
precipitation existed, the second cleavage of the
mixture avoided this step. The cleavage mixture of

30 TFA and water was diluted by additional water,
concentrated on an evacuated centrifuge and
lyophilized. HPLC evaluation of the mixture
demonstrated roughly equimolar representation of all
expected peaks.

35

6.2.2   DEPROTECTION OF N-TERMINAL FMOC
           GROUP AND ACETYLATION OF THE "FMOC
           BRANCH"

        Deprotection of the Fmoc group was followed by
acetylation of free N-terminal amino groups.
5 Acetylation was performed with a 0.3 M solution of N-
acetylimidazole in DMF for 20 min (ninhydrin test
negative). N-terminal Boc groups on the other branch
were deprotected after acetylation. Three randomly
chosen beads were sequenced and provided the following
10 readings (values in pmoles): 1st cycle: Y 213 (bead
1), G 161 (bead 2), Y 201 (bead 3), 2nd cycle: L 165
(1), Y 166 (2), Y 205 (3), 3rd cycle: Y 188 (1), L 128
(2), G 162 (3). The readings were not contaminated by
the amino acids present in the acetylated arm.
15   A part of the acetylated beads (about 100 mg)
were treated as described above (precipitation of the
mixture by ethyl ether and/or evaporation of cleavage
mixture and lyophilization) to reduce the handle and
cleave the acetylated peptides. HPLC analysis under
20 the same conditions have shown that during ether
precipitation a significant proportion of the library
was lost due to its solubility in ether. Evaporated
and lyophilized sample provided the same number of
peaks of approximately the same pattern as in the case
25 of the deprotected library, although retention times
shifted to higher values due to acetylation of the
Fmoc "branch".

6.2.3   REPLACEMENT OF THE BOC PROTECTING
30   GROUP WITH THE TFA GROUP

        The trifluoroacetyl group replaced the Boc group
at the N-terminus in order to permit a stepwise
sequencing experiment. First, the N-terminal Boc
group was cleaved from a resin sample (50 mg) while
35 the "Fmoc branch" was left protected. The free amino

groups were protected with trifluoroacetyl by
treatment with 10 equivalents (0.14 mmol, 21 μl) of
trifluroacetic acid anhydride in dichloromethane (0.5
ml) in the presence of DIEA (0.16 mmol, 28 μl). The

5   reaction was complete after 1h (ninhydrin test
negative). After the trifluoroacetylation, Fmoc group
on the other branch was removed and three beads were
submitted for sequencing. The sequence of the Fmoc
branch was determined. After sequencing the Fmoc

10   branch, the bead that was sequenced was removed from
the sequencer, and the bead was treated with a 0.2 M
solution of NaOH (3h, 20°C), dried, and submitted for
an additional three cycles of sequencing. The
appropriate sequences of the Boc branch predicted from

15   the sequencing of the Fmoc branch were obtained. 1st
bead (values in pmoles): 1. F (735), 2. V (643), 3. A
(837); after TFA removal: 1. Y (207), 2. L (187), 3. G
(76), sequence FVA/YLG; 2nd bead: 1. A (215), 2. A
(230), 3. F (193); after TFA removal: 1. G (88), 2. G

20   (86), 3. Y (80), sequence AAF/GGY; 3rd bead: 1. F
(63), 2. F (67), 3. V (41); after TFA removal: 1. Y
(15), 2. Y (12), 3. L (4), sequence FFV/YYL.

6.2.4 CLEAVAGE OF A PEPTIDE FROM ONE BEAD

25   Several beads of resin containing fully
deprotected sequences on the reduced SCAL handle were
placed separately into small glass vials and treated
overnight with 30 μl of neat TFA. Aliquots (3 μl)
were withdrawn and diluted with H_2O to the total volume

30   of 20 μl and analyzed by HPLC on microbore HPLC
(Michrom apparatus) (gradient 5-60% acetonitrile in
0.1% TFA in water over 20 min). Calculations based on
the average extinction coefficient of peptides at 215
run have shown that about 100-200 pmoles of peptide

35   was released from one polymeric bead.

6.3   DISCUSSION

The model encoded library is illustrated in
Figure 2. A branching linker attaches the synthetic
test compound peptide and the coding peptide to the
5   solid phase support. Generally, the synthetic test
compound can be a compound that does not undergo Edman
degradation, thus the sequence information from the
coding sequence provides for structure determination
of the test compound. Each of the subunits of the
10 synthetic test compound is unambiguously associated
with an amino acid in the coding arm, in a position-
specific fashion, thus allowing structure analysis.
Several approaches to building the coding
sequence exist. One procedure (Figure 1A) uses a
15 statistical distribution of both structures on the
polymeric bead. In this case, any possible ratio can
be achieved, and the possibility of producing a
cooperative effect of both sequences can be minimized.
In the second procedure (Figure 1B), both screening
20 and coding structures are built on the branched
attachment to the solid support, realized for example
by a diamino carboxylic acid (lysine). Both
"sequences" are present in the defined molar ratio and
a defined special arrangement accessible to the
25 acceptor molecule being screened. In the applications
where the release of the screened peptide into
solution is used, the localization of screening and
coding compound on the bead is of no concern, since
due to the use of different linkers, the coding
30 sequence is never released into the solution.
A simple scheme was pursued to conclusively
demonstrate the chemical synthesis of synthetic test
compound and coding sequences. A "synthetic test
compound" was built from A, F and V. These amino
35 acids were encoded with G, Y and L respectively in the

"coding" sequence. The synthetic test compound were
built on "test" branches, i.e., both amino groups of
lysine attached on another lysine side chain using
Fmoc chemistry (see Figure 2). Resin was split into
5   three parts and Nα-Fmoc protected amino acids were
coupled on the test branches and left in the protected
state. The corresponding Nα-Boc (coding) amino acids
were coupled on the encoding branch. All resin
support was mixed together and divided again into
10 three parts. Deprotection of the Nα-Fmoc group and
coupling of the subsequent Nα-Fmoc amino acid was
performed in the presence of Boc protection on the
other branch. The Boc protecting group is stable
under those conditions. In the next step, the Nα-Boc
15 group was cleaved and the Nα-Boc amino acid
corresponding to the Fmoc amino acid coupled to the
test branch was coupled on the coding branch in each
reaction. The procedure of mixing, splitting and
separate coupling of Fmoc and Boc amino acids was
20 repeated once more. The synthesis was performed on a
SCAL handle, which is stable under conditions of both
Boc and Fmoc strategies. This handle can, however, be
cleaved under relatively mild acidolytic conditions
after reduction of its sulfoxide moieties (Patek and
25 Lebl, 1991, Tetrahedron Lett. 32:3891-3894).
    Sequencing of beads prepared in this manner
demonstrated a molar ratio of 2:1 of screening to
coding sequence and the appropriate correspondence of
particular amino acids (Figure 2A). Using one aliquot
30 of beads, the "screening" sequence was acetylated and
clean sequence reading was obtained from the "coding"
sequence (Figure 2B). Using a different aliquot of
beads, the "coding" sequence was blocked by a
trifluoroacetyl group, sequencing of the "screening"
35 branch was performed, the trifluoroacetyl group was

cleaved from the sequenced beads and the sequence of the "coding" peptide was determined, confirming the results from the sequencing of the "screening" peptide (Figure 2C).

5   To verify that the synthetic strategy generates the predicted equimolar ratio of defined number of structures, the "minilibrary" represented in an aliquot was cleaved from the support. Reversed phase HPLC confirmed the presence of 27 different peptides.
10   The peaks identified in the trace were collected and submitted for sequence analysis, which confirmed the purity of each peptide and its composition. Cleavage of peptides was also performed from the single beads and the feasibility of analysis of peptides released
15   from only one bead was confirmed.

7.   EXAMPLE: NON-PEPTIDE LIBRARIES CODED BY A PEPTIDE STRUCTURE

This Example demonstrates that a peptide coding molecule can unambiguously encode a non-peptidyl test
20   compound when each is synthesized in parallel on a single bead. In this Example, the subunits of the test compound have been chosen so that each compound has a unique molecular weight. Comparing the observed molecular weight of the test compound to the sequence
25   of the coding peptide shows that a coding peptide codes for one test compound.

7.1 MATERIALS AND METHODS

Couplings of amino acids were performed by a
30   manual method using standard protocol at room temperature; protected amino acid (3 eq) in DMF was mixed with DIC (3 eq), or DIC and HOBT (3 eq each) with the resin and coupling was followed by analytical tests. Symmetric anhydrides were used where
35   specified.

The subunits used to prepare the non-peptide
library are shown in Scheme XII:

SCHEME XII

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1   2   3

10

4   5   6

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7   8   9

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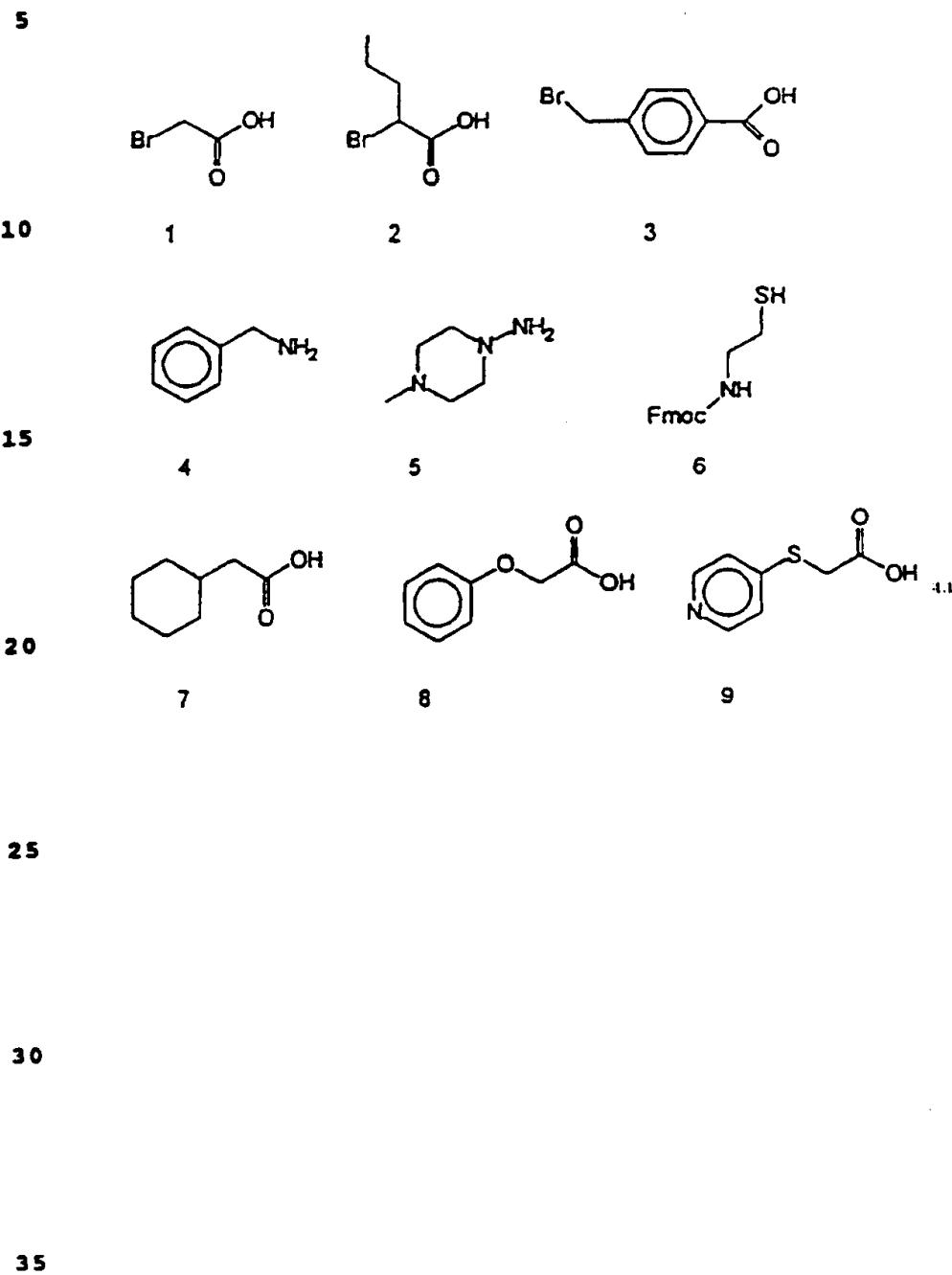
Fmoc-SCAL linker and Boc-Lys(Fmoc) were coupled
first to the resin (TentaGel S NH$_2$, 1g) using DIC and
HOBT. After cleavage of the Fmoc group, Fmoc-Trp was
coupled and the new Fmoc group was deprotected. The

5 peptide-resin was divided into three equal portions,
and three different bromoacids (one in each reaction
vessel, 3 eqs each) were coupled by the use of DIC in
DMF (3 eqs). The three acids were α-bromoacetic, α-
bromovaleric and bromotoluic acid. The coupling of

10 the last acid was repeated because of its low
reactivity, using a 6 fold excess of both acid and
DIC. Boc protection of the α-amino group of Lys was
removed by TFA and the first coding sequence Boc-
protected amino acids (Gly, Ala, Leu) were coupled by

15 DIC. Coding amino acids were chosen according to the
molecular weight of the non-peptide building blocks,
so the lightest block (bromoacetic acid in this case)
was coded by Gly, the heaviest one (bromotoluic) was
coded by Leu and medium one (α-bromovaleric) was coded

20 by Ala.
The three resin parts were pooled together,
washed thoroughly with DCM and deprotected by TFA/DCM
in preparation for the coupling of the next coding
amino acids. After deprotection, the resin was

25 divided again into three portions. Couplings of Boc-
protected amino acids (again Gly, Ala and Leu) were
performed as usual by means of DIC. After coupling
the coding peptide amino acids, the non-peptidyl
subunits were added. Two parts of the resin were

30 treated with 2M solutions of amines (benzylamine and
1-amino-4-methylpiperazine) in DMF overnight. The
third part was treated with 2M solution of
fluorenylmethyloxycarbonylaminoethylthiol, and after
the completion of the reaction the Fmoc group was

35

removed.   Coding of amines was based again on their
molecular weights.

The resin was pooled together once more, mixed
and divided into three portions for the final

5   couplings.   Carboxylic acids (cyclohexylacetic acid,
phenyloxyacetic acid and 4-pyridylthioacetic acid)
were coupled to the amines obtained (primary and
secondary) by DIC and the coupling reactions were
repeated twice using preformed symmetrical anhydrides

10 in 3-5 fold excess.   After obtaining a negative
chloranil test, the three batches of resin were
treated separately by TFA, neutralized, and the last
coding Boc-protected amino acids were coupled using
DIC and HOBT.   Coding of the last carboxylic acids was

15 based on the same scheme as before.   Finally all the
resin was pooled together.

Fast atom bombardment (FAB) mass spectroscopy
measurements were carried out on a ZAB EQ spectrometer
(VG Analytical Ltd, Manchester, UK). ^{1}H NMR spectra

20 were obtained on a General Electric QE 300 instrument.
Sequencing by Edman degradation was performed on an
ABI 4778 protein sequencer (Applied Biosystems, Foster
City, CA) and Porton PI 3010 instrument (Porton
Instruments, Tarzana, CA).   Both analytical and

25 preparative HPLC were carried out on a Waters 625 LC
system with a Waters 490E Programmable Multiwavelength
Detector using Vydac Peptide and Protein C18
analytical (0.46x250mm, 5μm, 1ml/min) and preparative
(10x250mm, 10μm, 3ml/min) columns, respectively.

30 Analyses of mixtures released from one bead were
performed on an Ultrafast Microprotein Analyzer
(Michrom BioResources, Pleasanton, CA) using a
Reliasil C18 column (5 μm, 300A, 1x150mm).   All
spectra are reported in ppm relative to

35 tetramethylsilane (δ) using either CDCl$_3$ or CD$_3$SOCD$_3$ as

solvents. UV/VIS absorption spectra were recorded on
a Hewlett Packard HP 8452A Diode-Array spectrophoto-
meter using a 1-cm quartz cuvette. Amino acid
analyses were carried out on a D-500 system (Durrum
5   Corp., Palo Alto, CA) system.

        7.2   RESULTS
            7.2.1   SYNTHESIS OF TWO FORMS OF ENCODED
                     LIBRARY
10   Two formats for the libraries have been completed
using this general approach. The only difference
between those two is the location of the SCAL linker
as shown in Scheme XIII.

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SCHEME XIII

$X_1 = CH_2$ .... $R_1 = H$ $X_2 = N-CH_2$

$X_1 = CHCH_2-CH_2-CH_3$ $R_1 = CH_3$ $X_2 = N-N$ $N-CH_3$

$X1 = -CH_2$ $R1 = CH-CH2-CH(CH3)2$ $X_2 = NHCH_2-CH_2-S$

$R_2 = CH_3$ $X_3 = -CH_2$ $R_3 = H$

$R_2 = CH-CH_2-CH(CH_3)_2$ $X_3 = O$ $R_3 = CH_3$

$R_2 = H$ $X_3 = S$ N $R_3 = CH-CH_2-CH(CH_3)_2$

The first library (A) contained the SCAL linker
on the N-ε of Lys, which was attached directly to
resin, and therefore Trp-amide was the last amino acid
in all the compounds of this library. In this

5   library, coding peptides remained on beads of resin
after cleavage. In the second library (B) the SCAL
linker was attached to the resin, and the last amino
acid in all compounds was Lys. Each of the compounds
released from this library included the synthetic

10 compound and coding sequence peptide.

    The non-amino acid building blocks used to
construct the synthetic test compound are shown above
in Scheme XII. These building blocks were chosen to
form unique molecular weight test compounds. The test

15 compounds, molecular weights and coding sequences are
shown in Table 1.

Table 1

Combination of Building Blocks Used
in the Construction of a Model Nonpeptide Library

| | M.W. | | Coding |
Combination	No Coding	With Coding*	Sequence
167	444.6	743.9	GGG
168	454.5	767.9	AGG
169	471.6	827.0	LGG
147	474.6	787.9	GAG
148	484.5	811.9	AAG
149	501.6	871.1	LAG
157	482.6	838.0	GLG
158	492.6	862.0	ALG
159	509.6	921.2	LLG
267	486.7	800.0	GGA

	268	496.6	824.0	AGA
	269	513.7	883.1	LGA
	247	516.7	844.1	GAA
	248	526.6	868.0	AAA
5	249	543.7	927.2	LAA
	257	524.7	894.1	GLA
	258	534.7	918.1	ALA
	259	551.7	977.3	LLA
10				
	367	520.7	876.1	GGL
	368	530.6	900.1	AGL
	369	547.7	959.2	LGL
	347	550.7	920.1	GAL
15	348	560.6	944.1	AAL
	349	577.7	1003.3	LAL
	357	558.7	970.2	GLL
	358	568.7	994.2	ALL
	359	585.7	1053.3	LLL
20				

* M.W. of branched compound containing the test compound and the coding peptide.

Beads from the first library (Scheme XIII, Upper Panel) were treated with reducing agent and individual beads were picked up for separate cleavage and sequence analyses. Five beads were studied. After cleavage of the non-peptide part, the beads were successfully sequenced (see Table 2) and the structure of the non peptide compound could be deduced. Solutions containing the cleaved compounds were analyzed on micro HPLC system.

Table 2

			Amino Acid Detected (pmol)		
Bead No.	Sub-unit Combin- ation	M.W. (m/z)	1st Cycle	2nd Cycle	3rd Cycle
1	149	501.6	L (50)	A (55)	G (72)
2	169	471.6	L (34)	G (31)	G (29)
3	258	534.7	A(101)	L (83)	A (98)
4	147	474.6	G (45)	A (41)	G (25)
5	157	444.6	G (39)	G (30)	G (22)

A sample (800 mg) of the second library (Scheme XIII, Lower Panel) was treated with 95% TFA after reduction of the SCAL linker, freeze-dried, dissolved in water, and separated on a semi-preparative HPLC column to 44 peaks using a gradient of 0-60% acetonitrile in 0.1% TFA in water over 200 min. Fractions were lyophilized and several peaks were analyzed by FAB MS and sequencing to show the correspondence between the structure predicted from the amino acid coding sequence and the molecular weight of the construct. Examples of peaks chosen at random for further analysis follows: Peak 4: RT 25.31 min, sequencing: 1. Leu (364 pmol), 2. Gly (139), 3. Gly (422); FAB MS - 827.0 (building block combination 169); Peak 8: RT 28.69 min, sequencing: 1. Gly (261), 2. Leu (176), 3. Ala (225); FAB MS - 770.2 (building block combination 257 w/o block 7); Peak 13: RT 31.47 min, sequencing: 1. Leu (792), 2. Leu (551), 3. Gly (128); FAB MS - 921.0 (building block combination 159); Peak 14: RT 32.27 min, sequencing: 1. Leu (7930), 2. Gly (1810), 3. Ala (1763); FAB MS - 883.0

(building block combination 269); Peak 15: RT 32.77
min, sequencing: 1. Leu (784), 2. Ala (447), 3. Ala
(360); FAB MS - 776.2 (building block combination 249
w/o block 9); Peak 16: RT 33.16 min, sequencing: 1.

5   Leu (1286), 2. Ala (918), 3. Ala (688); FAB MS - 776.2
(building block combination 249 w/o block 9); Peak 17:
RT 33.51 min, sequencing: 1. Leu (298), 2. Leu (280),
3. Ala (202); FAB MS - 826.2 (building block
combination 259 w/o block 9); Peak 19: RT 34.80 min,

10 sequencing: 1. Leu (641), 2. Gly (412), 3. Ala (460);
FAB MS - 883.1 (building block combination 269); Peak
20: RT 36.66 min, sequencing: 1. Leu (150), 2. Leu
(119), 3. Leu (80); FAB MS - 902.2 (building block
combination 359 w/o block 9); Peak 26: RT 41.77 min,

15 sequencing: 1. Gly (39), 2. Gly (38), 3. Gly (23); FAB
MS - 744.1 (building block combination 167); Peak 31:
RT 48.86 min, sequencing: 1. Ala (180), 2. Gly (98),
3. Ala (106); FAB MS - 824.0 (building block
combination 268); Peak 32: RT 49.46 min, sequencing:

20 1. Leu (234), 2. Leu (320), 3. Ala (277); FAB MS -
826.1 (building block combination 259 w/o block 9);
Peak 33: RT 50.70 min, sequencing: 1. Gly (152), 2.
Gly (120), 3. Ala (94); FAB MS - 800.1 (building block
combination 267).

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7.2.2   SYNTHESIS OF REPRESENTATIVE
          COMPOUNDS FROM THE NON-PEPTIDE LIBRARY

A component of the first library (A), compound I:
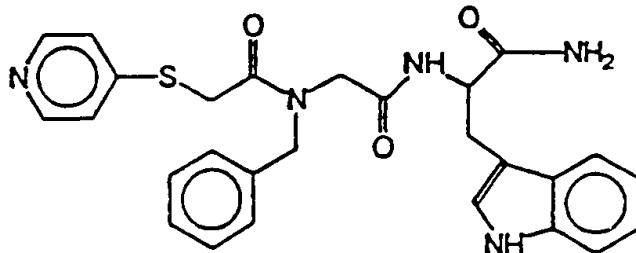
was synthesized on 0.23 g of Knorr resin (0.5 meq/g).
Fmoc-Trp was coupled first according to the general
protocol, using DIC and HOBT. After deprotection of
the amino group, α-bromoacetic acid (50 mg) was
coupled using DIC (50 μl) in DMF (0.5 ml).
Benzylamine (100 μl) was dissolved in 0.5 ml of DMSO
and bromoresin was treated with this solution
overnight. Final carboxylic acid, 4-pyridylthioacetic
acid (80 mg), was dissolved in 0.85 ml of DMPT and
preactivated with DIC (80 μl) and HOBT (80 mg) and
coupled to aminoresin for 10 hours. Coupling was
repeated using PyBrop and DIEA for activation.
Cleavage of the compound I was achieved in 95% TFA.
After cleavage, TFA was evaporated in vacuo and the
residue was dissolved in 30% aqueous acetonitrile and
lyophilized. The product obtained after drying was
redissolved in neat acetonitrile and precipitated by
ether. This operation was repeated twice and an
almost white precipitate was obtained. The product
showed two peaks on reverse phase (RP) HPLC. The
second peak gave the expected mass-spectrum of

compound I. The yield of component I after
purification on semipreparative RP HPLC was 18 mg.
Formula: $C_{27}H_{27}N_5O_3S$, MS expected 501.6, MS found - 502.2
$(M+H)^+$. ^{1}H NMR data (DMSO-d6): 10.804 d (1H, $N^\omega H$) ; B.
49 d (2H, pyridyl C_2H and C_6H); 8.35 d (1H, NH); 7.62 d
(2H, pyridyl C_3H and C_5H) ; 6.9 - 7.7 mm (Bzl and Trp
aromatic protons); 4.59 m (1H, Trp $C^\omega H$) ; 3.75 - 4.65 m
(aliphatic protons); 3.19 dd and 2.91 dd (2H, Trp $C^\beta H$).

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A second component of the first library (Scheme XII, A), compound II:

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10
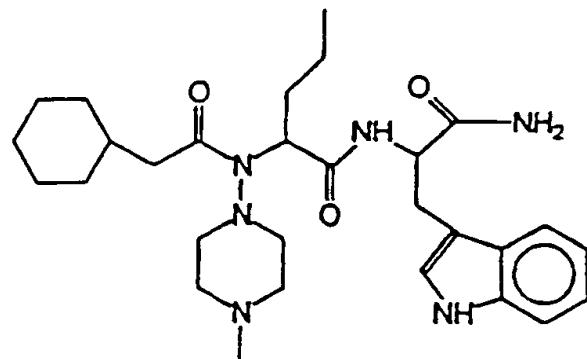
15 was synthesized according to the same scheme as compound I, using as subunits α-bromovaleric acid (40 μl), 4-methyl-aminopiperazin (100 μl) and cyclohexylacetic acid (80 mg). Formula: $C_{29}H_{44}N_6O_3$, MS expected - 524.7, MS found - 525.3 $(M+H)^+$, 558.2 **20** $(M+Na)^+$ and 573.2 $(M+K)^+$.

The third component of the first library (A), compound III:

25

30
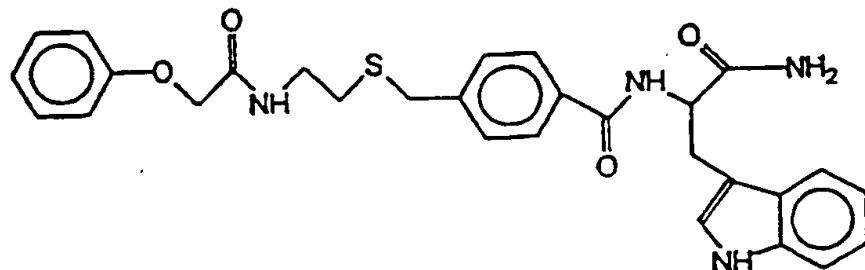
was synthesized according to the similar scheme as **35** compound I, using as building stones α-bromotoluic

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acid (120 mg), fluorenylmethyloxycarbonyl-
aminoethylmercaptan (280 mg) (deprotection after
coupling with piperidine/DMF) and phenoxyacetic acid
(80 mg).   Formula: $C_{29}H_{30}N_4O_4S$, MS expected 530.6, MS
5   found - 553.0 $(M+Na)^+$.

7.3   DISCUSSION
    This example demonstrates the ability to
construct nonpeptide structures in parallel with the
10   coding sequence. The difference in the libraries was
in the placement of the SCAL linker, allowing for the
selective cleavage of the product. In the first case,
(Scheme XIII Upper Panel), the cleavage of the linker
leads to the release of the non-peptide compound X_3-X_2-
15   X_1-Trp (Trp is attached for spectroscopic monitoring
purposes) connected to its coding peptidic structure
via a lysine moiety. Cleavage of the linker in the
second case (Scheme XIII, Lower Panel) leads to the
release of the non peptide compound X_3-X_2-X_1-Trp without
20   any attached coding peptide. Construction of the non
peptide compound involved (i) attachment of α-bromo
substituted carboxylic acid or bromomethylbenzoic acid
to the available amino group on the solid carrier,
(ii) alkylation of an amino (Zuckerman et al., 1992,
25   J. Am. Chem. Soc. 114:10646-10647) or thiol group of
an amine or N-protected aminomercaptan, and (iii)
acylation of a generated amino group by a derivative
of carboxylic acid. We have selected the building
blocks for this experiment in a manner which permits
30   the assignment of the structure of the constructed
screening molecules based solely on the molecular
weight of the construct (see Table I). Introduction
of every unnatural building block to the screening
structure was followed (or preceded) by the coupling
35   of a coding amino acid to the other arm of the

molecule. We have used only glycine, alanine and
leucine for coding (these amino acids therefore coded
a different structural element in every step of the
randomization). Assignment of these amino acids to
5 the particular structural element is given in Scheme
IX. Alkylation of amines or thiol used in this
experiment by 2-bromopentanoic acid attached to the
polymeric matrix lead to the generation of compounds
with a chiral center, therefore the number of
10 structural combinations is 36 rather than 27.
However, only 27 different bead types are generated
(with screening sequences of differing molecular
weights), 9 of which contain a mixture of
diastereoisomeric compounds. To simplify the analysis
15 of the mixtures and to demonstrate the ability to
perform this type of synthesis on polymeric carrier,
three of the possible structures were resynthesized as
individual compounds, using the same chemistry and
polymeric support as in the synthesis of the model
20 library.

The generated mixtures were cleaved from the
carrier after the reduction of the SCAL linker, and
analyzed by reversed phase HPLC. The number of peaks
obtained corresponds approximately to the predicted
25 number of 36. Individual peaks from the first type of
library were collected. Part of each fraction
collected was subjected to Edman degradation and part
was analyzed by mass spectroscopy. Results obtained
confirm the correlation of sequence determination with
30 molecular weight determination by mass spectroscopy,
confirming the viability of the principle of coding by
peptide sequence (Table 2).

An alternative analysis was performed on randomly
selected beads from the second library. Individual
35 beads were treated with a reducing agent to labilize

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the SCAL linker and the non peptide structure was
cleaved by a TFA/water mixture. After this treatment,
the beads were successfully sequenced (see Table II)
and the structure of the non peptide compound could be
5   deduced. The cleaved compounds were analyzed on a
micro HPLC system.

8.   EXAMPLE: LIBRARY: XXXX-Lys(XXXX)-Lys(ZZ)-
        βAla-Gly-βAla-Gly-TG

10   The present Example demonstrates the use of a
coding peptide to encode a non-sequenable portion of a
peptide simultaneously with the sequenable portion of
the test compound peptide.

15   8.1   MATERIALS AND METHODS
        8.1.1   SYNTHESIS OF THE LIBRARY
        The library was synthesized according to the
following protocol. 1. Coupling of Fmoc-Lys(Boc) to H-
βAla-Gly-βAla-Gly-TG; 2. Fmoc cleavage; 3. Coupling of
Fmoc-Lys(Fmoc); 4. Boc cleavage; 5. After division of
20   resin into 9 portions and the following Ddz protected
amino acids were coupled in separate reactions:
A,D,I,K,M,N,S,T,V; 6. Fmoc cleavage; 7. Coupling of
nine Fmoc-protected amino acids: Y,G,F,L,H,P,Q,R,E (Y
was coupled to that part of resin that had already
25   attached A, etc.); 8. Resin combined and Ddz cleaved;
9. Repeat steps 5-7; 10. Fmoc cleavage; 11. Coupling
of nine Fmoc-protected amino acids: Y,G,F,L,H,P,Q,R,E;
12. Repeat steps 10-11; 13. Fmoc cleavage; 14.
Side-chain protecting groups and Ddz removed by
30   mixture K (King et al., 1990, Int. J. Pep. Protein
Res. 36:255-266).
        One bead was submitted to four cycles of Edman
degradation: 1st cycle: Arg (64), Ile (67); 2nd cycle:
35   Gly (45), Thr (14); 3rd cycle: Phe (42); 4th cycle:
Arg (35). Ile was Ddz protected for coupling and

found in the first cycle. It coded for Phe, which was
detected in the third cycle. In the second cycle Thr
was detected as the amino acid that had been coupled
Ddz protected. Arg, coded by Thr, was accordingly
5 found in the fourth cycle of sequencing.

8.1.2 SCREENING PROTOCOL OF THE LIBRARY

The peptide library was screened according to
published procedures (Lam and Lebl, 1992,
10 Immunomethods 1:11-15). The peptide beads were first
mixed with double-distilled water to remove the DMF.
After extensive washing with PBS (137 mM NaCl, 2.7 mM
KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2), the beads
were coated with 0.05% gelatin (w/v) to block any
15 nonspecific binding. The beads were then incubated
with a 1:100,000 dilution of streptavidin-alkaline
phosphatase at 2 mg/ml (Pierce; Rockford, IL) in 2x
PBS/Tween/gelatin (2x PBS, 0.1% Tween-20 (v/v), and
0.05% gelatin (w/v)). The beads were then thoroughly
20 washed with TBS (137 mM NaCl, 2.7 mM KCI, 25 mM Tris
base, pH 7.4) and the standard substrate 5-bromo-4-
chloro-3-indolyl phosphate was added. The beads,
together with the substrate, were then transferred to
petri dishes for color development. After 30 minutes
25 to 1 hour, the colored beads were collected, with the
aid of a micropipette, washed with 6M guanidine
hydrochloride, pH 1.0, and subjected to sequencing as
described.

The remaining library of colorless beads were
30 then recycled with 8M guanidine hydrochloride, pH 2.0,
thoroughly washed with PBS, and incubated with 60 pM
biotinylated anti-β-endorphin (clone 3-E 7, Boehringer
Mannheim) in 2 x PBS/Tween/gelatin overnight. After
thorough washing, streptavidin-alkaline phosphatase
35 was added. One hour later, the beads were washed,

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substrate was added, and color development proceeded
as described above. The color beads were then
physically isolated and subjected to sequencing. In
these two experiments, only the darkest beads were
5   sequenced.

8.2 RESULTS AND DISCUSSION

Sections 6 and 7, *supra*, show that a "test
compound" can be coded by a "coding" peptide sequence.
10 This principle can also be used for determination of
the structure of peptides containing a nonsequenceable
component within the peptide chain. In this case it
is necessary to code only for the amino acid residues
located on the carboxyl terminus of the molecule,
15 after the nonsequenceable part. We have constructed a
library mimicking this situation, although the "test
compound" does not actually contain a non sequenable
component. The structure of the library is given in
Scheme XIV.
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SCHEME XIV

5

$$Z_2Z_1$$
$$X_4X_3X_2X_1$$
$$X_4X_3X_2X_1—K—K—\text{(TG)}$$
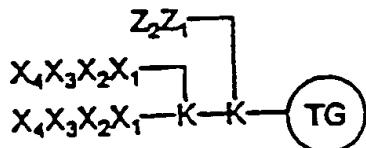
10

X = YGFLHPQRE

Z = AD IKMNSTV

15 Amino acid residues X_4 and X_3, in the "test compound"
    arm are not coded by any counterpart in the "coding"
    arm. Amino acids Z_1, and Z_2 code for residue X_1, and X_2
    and are present in one half the concentration of the
    amino acids in the "test" sequence. Two cycles of
20 Edman degradation can reveal the structure of the
    peptide of interest. The amino acid detected in the
    greater amount is the residue from position 1 or 2 of
    the "test" sequence. The amino acid detected in lower
    quantity is the residue coding position 4 or 3 of this
25 sequence. The coding amino acid may be the same as
    the one for which it is coding, or it can be a
    different one. The coding and screening set of amino
    acids used in this example are given in Scheme XIV.

30

Table 3

Sequencing Results

1st Cycle	2nd Cycle	Deduced Sequence	Target
Y,D	G,I	YGGF	anti-β-endorphin
Y,N	G,I	YGPF	anti-β-endorphin

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| Y,D | G,K | YGGL (3x) | anti-β-endorphin |
| H,S | P,I | HPQF (5x) | streptavidin |

5   The synthesis of the library was performed with
the use of a combination of three amino protecting
groups. Temporary protection of the α-amino group in
the "screening" sequence was provided by the Fmoc
group, which is cleavable by piperidine in
10   dimethylformamide. Temporary protection of the
"coding" sequence was achieved by the use of the Ddz
group (Birr et al., 1972, Liebig's Ann. Chem. 763:162-
173), cleavable with diluted trifluoroacetic acid
(2%). Side chain functional groups were protected by
15   tert-butyl type protecting groups cleavable by
trifluoroacetic acid of higher concentration (50%).
One cycle of randomization with sequence tagging
consisted of (i) division of resin into the number of
reaction vessels corresponding to the number of amino
20   acids randomized in this step, (ii) coupling Fmoc
protected amino acids (Y, G, F, L, H, P, Q, R, E),
(iii) washing, cleavage of Ddz group, and
neutralization, (iv) coupling of corresponding Ddz
protected amino acids (A, D, I, K, M, N, S, T, V), (v)
25   mixing the solid support and deprotection of the Fmoc
group.
       This library was used in the screening against
two model targets, anti-β-endorphin monoclonal
antibody, and streptavidin. Positive beads were
30   identified by the standard staining technique (Lam and
Lebl, 1992, Immunomethods. 1:11-15; Lam et al., 1991,
Nature 354:82-85), and the beads (5 for each target)
identified in this screen were subjected to two cycles
of Edman degradation. Results of two cycles of Edman
35   degradation are given in Table 3. As can be seen,

streptavidin positive beads gave in all cases H (X_1)
and S (Z_1) (coding for Q, X_3) in the first cycle, and P
(X_2) and I (Z_2) (coding for F, X_4) in the second cycle.
Therefore sequence of the screening arm HPQF could be

5   decoded easily. Beads identified in the anti-β-
endorphin screening gave more varied results. Besides
Y (X_1) and D (Z_1) (coding for G, X_3), N (Z_1) (coding for
P, X_3) was also found in the first cycle, and G (X_2)
and I (Z_2) (coding for F, X_4) and K (Z_2) (coding for L,

10   X_4) were found in the second cycle. Therefore
sequences YGGL (3x), YGGF, and YGPF could be
constructed from these data. These sequences are in
agreement with the data obtained earlier (Lam and
Lebl, supra; Lam et al., 1991, supra; Lam et al.,

15   1993, Bioorg. Med. Chem. Lett. 3:419-429).

These experiments clearly establish that peptides
can be used to encode other structures, which are
partially or completely unsequenable. This technology
will have major significance because of its broad

20   applicability to the study of ligand-acceptor
molecular interactions and to drug development.
Encoded libraries open the door to applying broad
parallel approaches to drug synthesis and screening
non-peptide libraries.

25

9.   EXAMPLE: LIBRARIES OF NON-PEPTIDE
STRUCTURES BASED ON SOLID
PHASE PEPTIDE SYNTHESIS CHEMISTRY

The present Example combines the simplicity of
synthesis of peptide structures with the diversity

30   available using alternative subunits besides standard
amino acids. The simplest subunits for the library
construction are discussed in Section 5.5.9, supra.

Synthesis of the present library involved use of
trifunctional amino acids and modification of a side

35   chain to achieve the structural multiplicity. Amino

acids like diaminobutyric acid, aspartic acid, cystine and/or iminodiacetic acid are the smallest subunits onto side chains of which carboxylic acids, amines, isocyanates or halides (aliphatic, aromatic,

5   heterocyclic) can be attached. These amino acids can themselves act as a scaffold for further derivatization.

To achieve a reasonable binding to an acceptor (e.g., receptor, antibody, enzyme, nucleic acid, etc.)

10 the appropriate spatial arrangement of the interacting structures must be realized. Linear presentation of amino acid side chains in peptide libraries may not be an optimal format for the selection of the best binding structures. The optimal strategy for

15 displaying the interacting structures may be their placement on a molecular scaffold, which would map the appropriate conformational space. Interrelationships of the same individual building blocks in the scaffolding arrangement can be varied using different

20 scaffolding as well as different side chains.

9.1 MATERIALS AND METHODS

9.1.1 INSTRUMENTS

Fast atom bombardment (FAB) mass spectroscopy

25 measurements were carried out on a ZAB EQ spectrometer (VG Analytical Ltd, Manchester, UK). ^{1}H NMR spectra were obtained on a General Electric (Fullerton, CA) QE 300 instrument. Sequencing by Edman degradation was performed on an ABI 4778 protein sequencer (Applied

30 Biosystems, Foster City, CA) and Porton PI 3010 instrument (Porton Instruments, Tarzana, CA). Both analytical and preparative HPLC were carried out on a Waters 625 LC system with a Waters 490E Programmable Multiwavelength Detector using Vydac Peptide and

35 Protein C18 analytical (4.6x250mm, 5μm, 1ml/min) and

preparative (10x250mm, 10μm, 3ml/min) columns,
respectively. Analyses of mixtures released from one
bead were performed on an Ultrafast Microprotein
Analyzer (Michrom BioResources, Pleasanton, CA) using

5 a Reliasil C18 column (5 μM, 300A, 1x150mm). All
spectra are reported in ppm relative to
tetramethylsilane (δ) using either CDCl₃ or CD₃SOCD₃ as
solvents. UV/VIS absorption spectra were recorded on
a Hewlett Packard HP 8452A Diode-Array

10 spectrophotometer using a 1-cm quartz cuvette. Amino
acid analyses were carried out on a D-500 system
(Durrum Corp., Palo Alto, CA) system.

9.1.2 PROCEDURES

15 Solid phase synthesis was performed manually in
polypropylene syringes as described by Krchnak and
Vagner (1990, Peptide Res. 3:182-193). Syntheses were
performed on TentaGel S NH₂ (TG) resin (Rapp Polymere,
Tubingen, Germany, 130 or 80μm, 0.23mmol/g) modified

20 with SCAL handle (Patek and Lebl, 1991, Tetrahedron
Lett. 32:3891-3894) (safety-catch amide linker) or
with an appropriate linker. Fmoc protecting groups
were cleaved with 50% piperidine/DMF for 1x10min, Tfa
groups by repeated treatment (3x 1 min + 90 min) with

25 10% piperidine/water. Npys goups were removed by 0.3M
solution of HCl in dioxane for 5 + 30 min, Aloc group
by (Ph₃P)₄Pd in DMF/AcOH/N-Me-Morpholine (10:2:1), Boc
groups were cleaved with 30% TFA/DCM containing 3% of
anisole for 20min. A solution of DIEA/DCM (10%) was

30 used for neutralization after Boc cleavage. A mixture
of BOP/HOBt/DIEA (1:1:2eq) in DMF was used for the
activation of both Nα-Fmoc and Boc amino acids. The
completeness of each condensation reaction (1.5 - 40
hrs) was checked by ninhydrin test, or by chloranil

35 test in the cases of coupling to secondary amino

groups. The coupling protocol included washing with
DMF (6-8 times) [followed by washing with DCM in case
of Boc protected amino acids] between coupling and
deprotection and between deprotection and coupling.

5   The SCAL linker was reduced by 20% $(EtO)_2P(S)SH$ in DMPU
for 2 hours. Final cleavage was done by 95% TFA - 5%
water mixture.

9.1.3 REAGENTS

10   Commercial-grade solvents were used without
further purification. Protected amino acids were
obtained from Bachem (Torrance, CA), Advanced ChemTech
(Louisville, KY), or Propeptide (Vert-le-Petit,
France). Amines and carboxylic acids were obtained

15   from Aldrich (Milwaukee, WI).

9.1.4   SYNTHESIS OF A NONPEPTIDIC LIBRARY ON NONPEPTIDIC SCAFFOLDING

Mono tert.butyloxycarbonylethylenediamine. This
compound was prepared as described previously (Krapcho

20   et al., 1990, Synthetic Commun. 20:2559-2564).
Briefly, a solution of tert.butyl dicarbonate (5.0 g,
0.023 mol) in dioxane (50 ml) was slowly added to
ethylenediamine (11.0 ml, 0.165 mol) in dioxane (60

25   ml). After 24 hrs of stirring the solvent was
evaporated and the residue was dissolved in water (80
ml), insoluble byproduct was filtered off and the
filtrate was extracted with dichloromethane (3x100
ml). After evaporation of the solvent the product was

30   crystallized from solvent mixture of diethylether -
petroleum ether or ethyl acetate - petroleum ether.
Yield 2.6 g (71%). NMR (300 MHz, DMSO-d_6, 25°C) d: 1.39
(s, 9H, tBu), 2.83 (m, 2H, C^aH_2), 3.16 (q, 2H, C^bH_2),
6.93 (t, 1H, NH), 7.77 (br, 2H, NH_2). M.p. 75°C

35

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N-tert.butyloxycarbonyl-N'-
fluorenylmethyloxycarbonylethylenediamine. A solution
of fluorenylmethyl succinimidyl carbonate (31.0 g,
0.092 mol) in acetonitrile (300.0 ml) was slowly added

5   to mono tert-butyloxycarbonylethylenediamine (10.0 g,
0.063 mol) dissolved in 10% aq. Na_2CO_3 (250 ml).
Acetonitrile was evaporated and the product was
extracted with ethyl acetate, organic phase was dried
over Na_2CO_3, concentrated and the product was allowed

10 to crystallize by adding petroleum ether. The product
was collected on filter, washed with petroleum ether.
Yield 20.0 g (84%). TLC petroleum ether -
diethylether 88:12 R_f 0.64. NMR (300 MHz, DMSO-d_6,
25°C) d: 1.37 (s, 9H, tBu), 2.99 (m, 4H, CH_2CH_2), 4.20-

15 4.29 (m, 3H, Fmoc OCH_2CH-), 6.76 (t, 1H, NH), 7.25 (t,
1H, NH), 7.33-7.89 (m, 8H, Fmoc). M.p. 146-148°C.

Mono N-fluorenylmethyloxycarbonylethylenediamine
trifluoroacetate. N-tert-butyloxycarbonyl-N'-
fluorenylmethyloxycarbonylethylene-diamine (20.0 g,

20 0.052 mol) was treated with trifluoroacetic acid and
anisole in dichloromethane (10:10:1) for 1 hour at
room temperatute. After evaporation to dryness the
crude product was crystallized from solvent mixture of
ethyl acetate-n-hexane. Yield 15.0 g (73%). NMR (300

25 MHz, DMSO-d_6, 25°C) d: 2.85 (m, 2H, C^aH_2), 3.22 (m, 2H,
C^bH_2), 4.23-4.36 (m, 3H, Fmoc OCH_2CH-), 7.37 (m, 1H,
NH), 7.34-7.89 (m, 8H, Fmoc), 7.77 (br, 2H, NH_2). M.p.
128-129°C.

cis,cis-1,3,5-Trimethylcyclohexane-5-carbocylic
30 acid-1,3-dicarboxylic anhydride (1). Compound (1) was
prepared as described (Askew et al., 1989, J. Am.
Chem. Soc. 111:1082-1090). Briefly; cis,cis-1,3,5-
trimethylcyclohexane-1,3,5-tricarboxylic acid (1.0 g,
0.004 mol) was refluxed in xylene (50 ml) for 19 h
35 under nitrogen using Firestone valve and Dean-Stark

trap. The resulting solution was concentrated in
vacuo and the product was allowed to crystallize.
After collection on filter the product was dried in
vacuo at 70°C for 1 hour. Yield 0.78 g (82%). NMR (300
5   MHz, DMSO-d_6, 25°C) d: 1.10 (s, 3H, CH_3), 1.16 (s, 6H,
2CH_3), 1.33, 2.39 (d,d, 4H, 2CH_2), 1.33, 2.15 (d,d, 2H,
CH_2), 12.60 (s, 1H, COOH). M.p. 252-253°C, lit.(32)
m.p. 252-254°C.

      5-(N-tert.Butyloxycarbonylaminoethylcarboxamide)-
10 cis,cis-1,3,5-trimethylcyclohexane-1,3-dicarboxylic
acid (2). Acid anhydride (1) (0.5 g, 0.002 mol) was
dissolved in DMF (4 ml) and mono
tert.butyloxycarbonylethylenediamine (0.33 g, 0.002
mol) dissolved in DMF (4 ml) was added under nitrogen.
15 Reaction mixture was stirred about 5 hours (TLC
monitoring) and then DMF was evaporated. The product
was crystallized from solvent mixture of ethyl acetate
- petroleum ether. Yield 0.38 g (47%). NMR (300 MHz,
DMSO-d_6, 25°C) d: 1.08 (s, 3H, CH_3), 1.13 (s, 6H, 2CH_3),
20 1.34 (s, 9H, tBu), 1.07, 2.51 (d,d, 4H, 2CH_2), 2.43
(d, 2H, CH_2), 2.96 (m, 4H, 2CH_2), 6.70 (t, 1h, NH),
7.71 (t, 1H, NH), 12.10 (s, 1H, COOH). M.p. 161-164°C.

      5-(N-tert.Butyloxycarbonylaminoethylcarboxamide)-
cis,cis-1,3,5-trimethylcyclohexane-1,3-dicarboxylic
25 anhydride (3). Dicyclohexylcarbodiimide (0.55 g,
0.002 mol) was added to the solution of diacid (2)
(1.0 g, 0.0025 mol) in DCM (70 ml) under nitrogen.
After 4 hrs of stirring the reaction mixture was
concentrated and dicyclohexylurea was filtered off.
30 The filtrate was evaporated to dryness and the residue
was crystallized from solvent mixture of ethyl acetate
- petroleum ether, dried in dessicator (KOH, P_2O_5) in
vacuo. Yield 0.9 g (95%)

      5-(N-tert.butyloxycarbonylaminoethylcarboxamide)-
35 3-(N-fluorenylmethyloxycarbonylaminoethylcarboxamide)-

cis,cis-1,3,5-trimethylcyclohexane-1-carboxylic acid
(4). The anhydride (3) (0.85 g, 0.002 mol) was
dissolved in DMF (10 ml) and solution of mono N-
fluorenylmethyloxycarbonylethylene-diamine
5 trifluoroacetate (0.88 g, 0.002 mol) in DMF (15 ml) in
the presence of triethylamine (pH 8.5 adjusted) was
added under nitrogen. Reaction mixture was stirred
for 4 hrs (TLC monitoring) and then evaporated to
dryness. The crude product was flash chromatographed
10 (Silica Gel Merck 60 230-400 mesh) in solvent system
DCM-MeOH 25:1. Fractions containing pure product were
combined, evaporated to dryness and the product was
crystallized from solvent mixture of ethyl acetate -
petroleum ether. Yield 0.32 g (24%). NMR (300 MHz,
15 DMSO-d_6, 25°C) d: 1.08 (s, 6H, 2CH$_3$), 1.14 (s, 3H, CH$_3$),
1.35 (s, 9H, tBu), 4.25 (m, 3H, Fmoc OCH$_2$CH-), 6.86 (t,
1H, NH), 7.32-7.89 (m, 8H, Fmoc). M.p. 118-121

      Non-peptidic library on non-peptidic scaffolding.
The compound (4) was used as scaffolding for a
20 nonpeptidic library. TentaGel S NH$_2$ (0.4 g,
substitution 0.21 mequiv NH$_2$/g) was subjected to solid
phase synthesis using the following protocol:

Step	Reagent	Time
1	5% DIEA/DMF	2x5 min
2	DMF wash	10x2 min
3	Fmoc-Lys(Boc)/BOP	until ninhydrin test is negative
4	repeat step 2	
5	DCM	10x2 min
6	TFA/DCM/anisole	1x30 min
7	repeat step 5	
8	5% DIEA/DCM	3x2 min
9	repeat step 2	

| 10 | resin was divided into 20 portions and acids defined below coupled. Anhydrides were used as such, other carboxylic acids were coupled using BOP reagent. |
| | |

5

pivalic acid
phenylacetic acid
diphenylacetic acid
1-adamantaneacetic acid
Z-Gly
ClZ-β-Ala
10
ClZ-aminocaproic acid
diBoc-quanidinoacetic acid
diBoc-ϵ-quanidinopentanoic acid
succinamic acid
2-furoic acid
p-hydroxybenzoic acid
isonicotinic acid
15
4-phenylbutyric acid
acetic anhydride
n-butyric anhydride
n-caproic anhydride
benzoic anhydride
succinic anhydride
glutaric anhydride

20

11	repeat step 2	
12	50% piperidine/DMF	10 min
13	repeat step 2	
14a	compound 4/DIC/HOBt	30 min (preactivation)

25

14b	product of step 14a	until Kaiser test is negative
15	repeat step 11-13	
16	resin divided into 20 portions and acids defined in step 10 coupled. Anhydrides were used as such, other carboxylic acids were first preactivated by DIC/HOBt mixture.	

30

| 17 | DMF wash | 10x2 min |
35 | 18 | DCM | 3x2 min |

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19	TFA/DCM/anisole	1x30 min
25	DCM	5x2 min
26	5% DIEA/DCM	3x2 min
27	DMF wash	4x2 min
28	resin divided into 20 portions and acids defined in step 10 coupled. Anhydrides were used as such, other carboxylic acids were first preactivated by DIC/HOBt mixture.	
29	DMF wash	10x2 min
30	DCM wash	3x2 min
31	TFA/TFMSA/TA	30 min
32	DCM wash	3x2 min
33	DMF wash	5x2 min

9.1.5 BRANCHED LIBRARY ON TENTAGEL

TentaGel S NH$_2$ (5g, 0.23 mmol/g, 130 μm bead size) was preswollen in DMF and the branched library was built according to the following protocol: (1) Coupling of SCAL linker; (2) Deprotection of Fmoc; (3) Coupling of Fmoc-Lys(Tfa); (4) Deprotection of Fmoc; (5) Coupling of Fmoc-β-Ala; (6) Deprotection of Fmoc; (7) Coupling of Fmoc-Lys(Boc); (8) Deprotection of Fmoc; (9) Coupling of Fmoc-β-Ala; (10) Deprotection of Boc; (11) First randomization; (12) Deprotection of Fmoc; (13) Second randomization; (14) Deprotection of Tfa; (15) Coupling of Fmoc-Lys(Tfa); (16) Deprotection of Fmoc; (17) Coupling of Fmoc-β-Ala; (18) Deprotection of Fmoc; (19) Third randomization; (20) Deprotection of Tfa; (21) Fourth randomization; (22) Deprotection of side-chains. In each randomization the following acids were coupled: acetic, n-butyric,

pivalic, n-caproic, benzoic, phenylacetic,
4-phenylbutyric, diphenylacetic, 1-adamantaneacetic,
succinic, glutaric, glycine, β-alanine,
epsilon-amino-n-caproic, guanidoacetic,
5   gamma-guanidinobutyric, succinamic, p-hydroxybenzoic,
2-furoic, and isonicotinic acid. These subunits, and
the corresponding amino acid code for each subunit,
are listed in Table 4. Amino acids were coupled as
anhydrides when commercially available (10 eq of
10   anhydride, 1.2 eq of DIEA), or they were preactivated
for 20 min (acid 12 eq, DIC 10 eq, HOBt 10 eq). Due
to their very poor solubility in DMF, guanidino acids
(12 eq) were dissolved in DMF containing HOBt and LiCl
and activated by DIC. However, in a control experiment
15   only ca 70 to 80% coupling was observed under those
conditions. After finishing the synthesis the library
was washed with TFA (3x), DCM (5x), DMF (5x),
DMF/0.1%HCl (1:1) (3x), and 0.02% HCl.

20

Table 4

Protection, Activation, and Coding Scheme for Acids

Acid	Prot.	Activ.	Coded By
acetic acid		anhydr.	Ala
n-butyric acid		anhydr.	Asn
pivalic acid		anhydr.	Asp
n-caproic acid		anhydr.	Glu
benzoic acid		anhydr.	Gln
phenylacetic acid		DIC,HOBt	Gly
4-phenylbutyric acid		DIC,HOBt	His
diphenylacetic acid		DIC,HOBt	Ile
1-adamantaneacetic acid		DIC,HOBt	Leu
succinic acid		anhydr.	Lys
glutaric acid		anhydr.	Met

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glycine	Boc	DIC,HOBt	Orn
beta-alanine	Boc	DIC,HOBt	Phe
ε-amino-n-caproic acid	Boc	DIC,HOBt	Pro
guanidoacetic acid	diBoc	DIC,HOBt	Ser
γ-guanidinopentanoic acid	diBoc	DIC,HOBt	Thr
succinamic acid		DIC,HOBt	Trp
p-hydroxybenzoic acid		DIC,HOBt	Tyr
2-furoic acid		DIC,HOBt	Val
isonicotinic acid		DIC,HOBtl	Nva

9.1.6 BRANCHED ENCODED LIBRARY ON TENTAGEL

TentaGel S NH$_2$ (5g, 0.23 mmol/g, 130 μm bead size) was preswollen in DMF and the branched library with coding sequence was built according to the following protocol: (1) Coupling of Fmoc-Lys(Ddz-Gly); (2) Deprotection of Fmoc; (3) Coupling of Fmoc-Lys(Tfa); (4) Deprotection of Fmoc; (5) Coupling of Fmoc-β-Ala; (6) Deprotection of Fmoc; (7) Coupling of Fmoc-Lys(Npys); (8) Deprotection of Fmoc; (9) Coupling of Fmoc-β-Ala; (10) Deprotection of Fmoc; (11) First randomization; (12) Deprotection of Ddz in each reaction vessel separately; (13) Coupling of Fmoc-protected coding amino acid; (14) Deprotection of Npys; (15) Second randomization; (16) Deprotection of Fmoc in each reaction vessel separately; (17) Coupling of Fmoc-protected coding amino acid; (18) Deprotection of Fmoc from coding arm; (19) Coupling of Ddz-Phe; (20) Deprotection of Tfa; (21) Coupling of Fmoc-Lys(Npys); (22) Deprotection of Fmoc; (23) Coupling of Fmoc-β-Ala; (24) Deprotection of Fmoc; (25) Third randomization; (26) Deprotection of Ddz in each reaction vessel separately; (27) Coupling of Fmoc-protected coding amino acid; (28) Deprotection of

Npys; (29) Fourth randomization; (30) deprotection of
Fmoc in each reaction vessel separately; (31) Coupling
of Fmoc-protected coding amino acid; (32) Deprotection
of Fmoc; (33) Deprotection of side-chains. The
5   library was washed with TFA (3x), DCM (5x), DMF (5x),
DMF/0.1%HCl (1:1) (3x), and 0.02% HCl.

9.1.7   BRANCHED LIBRARY WITH CODING ON FAST FLOW SEPHAROSE (FFS)

10   FFS was sieved to obtain more narrow particle
size distribution (bead size 85-125 um), placed in a
reaction vessel for peptide synthesis and washed 10
times with DMF. Fmoc-Gly (2.97 g) in DMF was
activated by DIC (1.57 ml) and HOBt (1.35 g) and added
15 to 10 ml of FFS. The reaction was catalyzed by 0.25 g
of dimethylaminopyridine and the suspension was shaken
overnight. Fmoc-Gly-FFS was washed 10 times with DMF,
Fmoc was cleaved, resin was washed with DMF and the
substitution calculated according to absorbance of
20 deprotecting solution at 302 nm. Typical substitution
was 0.1 mmol/ml. Then mixture of Fmoc-β-Ala and Boc-
β-Ala (molar ratio 3:1) was activated by DIC and HOBt
and coupled to Gly-FFS in 3 molar excess. FFS was
washed 10 times with DMF, Fmoc groups removed and free
25 amino groups acetylated with Ac$_2$O/Py (1:1) for 10 min.
After washing 10 times with DMF and DCM, Boc was
removed by TFA/DCM/anisole (45:45:10) for 5+30 min,
FFS was washed with DCM (10 times), neutralized with
2% DIEA/DCM (3 times 1 min) and washed 10 times with
30 DMF.
        The branched library with coding sequence was
built according to the following protocol: (1)
Coupling of Fmoc-Lys(Ddz-Gly); (2) Deprotection of
Fmoc; (3) Coupling of Fmoc-Lys(Alloc); (4)
35 Deprotection of Fmoc; (5) Coupling of Fmoc-β-Ala; (6)
Deprotection of Fmoc; (7) Coupling of Fmoc-Lys(Npys);

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(8) Deprotection of Fmoc; (9) Coupling of Fmoc-β-Ala;
(10) Deprotection of Fmoc; (11) First randomization;
(12) Deprotection of Ddz in each reaction vessel
separately, (13) Coupling of Fmoc-protected coding

5 amino acid, (14) Deprotection of Npys; (15) Second
randomization; (16) Deprotection of Fmoc in each
reaction vessel separately, (17) Coupling of Fmoc-
protected coding amino acid, (18) Deprotection of Fmoc
from coding arm, (19) Coupling of Ddz-Phe, (20)

10 Deprotection of Alloc; (21) Coupling of Fmoc-
Lys(Npys); (22) Deprotection of Fmoc; (23) Coupling of
Fmoc-β-Ala; (24) Deprotection of Fmoc; (25) Third
randomization; (26) Deprotection of Ddz in each
reaction vessel separately, (27) Coupling of Fmoc-

15 protected coding amino acid, (28) Deprotection of
Npys; (29) Fourth randomization; (30) deprotection of
Fmoc in each reaction vessel separately, (31) Coupling
of Fmoc-protected coding amino acid, (32) Deprotection
of Fmoc, (33) Deprotection of side-chains. The

20 library was washed with TFA (3x), DCM (5x), DMF (5x),
DMF/0.1%HCl (1:1) (3x), and 0.02% HCl.

9.1.8   LIBRARY OF MIXED PEPTIDE AND NON-PEPTIDE SUBUNITS

25 The library was synthesized on TentaGel S NH$_2$, 90
μm, (Rapp Polymere, Germany) (2g, substitution
0.25mmol/g, 0.5 mmol). First, the sequence Gly-βAla-
Gly-βAla-Gly-Lys(Tfa)-TG identical for all sequences
was synthesized using 5 eq excess of activated Fmoc-

30 amino acids (DIC/HOBt activation). After the N-
terminal Fmoc-deprotection, the resin was divided into
9 portions in the ratio 17:8:5:1:1:1:1:1:1 (36 parts,
0.014 mmol per part). The structures of the subunits
used in this library are shown in Figure 8, along with

35 the amino acid dipeptide code for each of the
subunits. Subunits that were added to each portion

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are indicated in parentheses. These portions were
further treated as follows:

    17-part portion (X_2= 1-17): Fmoc-Dab(Boc)-OH was
coupled (5 eq excess), the Boc-side chain protecting
5   group was removed by TFA/DCM/anisole (50:50:2, 15 min)
and after the washing steps this portion was divided
into 17 parts. Corresponding acids were coupled to
the free side chain amino group by standard DIC/HOBt
activation.

10   **8-part portion (X_2 = 18-25)**: Fmoc-Asp(OBu^t)-OH
was coupled (5 eq excess), the Bu^t-side chain
protecting group was removed by TFA/DCM/anisole
(50:50:2, 15 min) and after the washing steps this
portion was divided into 8 parts. Corresponding
15   amines were coupled to the side chain according to the
procedure described below.

    5-part portion (X_2= 30-34): A solution of Fmoc-
IDA-anhydride (10 eq, 0.7 mmol in 2 ml DMF) was added
to the resin and shaken for 30 min. The procedure
20   was repeated once more, the resin washed with DMF and
divided into 5 parts. Amines were coupled according
to the following procedure.

    The general procedure for coupling of amines in
the side chain, used for linking the structures 18-25
25   and 30-34, follows: The resin bound carboxy-groups
(0.014 mmol per part) were activated by a mixture of
DIC/HOBt (10 eq) in 0.4 ml DMF for 30 min. This
mixture was removed without washing and 30 eq of the
appropriate amine in 0.2 ml DMF were added. In the
30   case of p-toluidine, equimolar amount of DIEA was
added for the neutralization of the hydrochloride.
The resin was shaken for 1 hour and washed with DMF.

    1-part (single) portions (X_2 = 26-29, 35, 36):
The N-Fmoc-protected monoamides of iminodiacetic acid
35   (for structures 26-29) (10 eq, 0.014 mmol per part)

and Fmoc-Cys(Bzl)-OH and Fmoc-D-Pen(Bzl)-OH (5 eq,
0.07 mmol) were coupled by DIC/HOBt activation to the
single portions, respectively.

5   **Further procedures for all 36 portions:** The N-
terminal Fmoc-group was cleaved by piperidine/DMF. To
the subunits 1-25, 35, 36, Boc-Gly-OH was coupled by
DIC/HOBt activation (5 eq, 0.07 mmol per part).
Subunits 26-34 were reacted with symmetrical anhydride
of Boc-Gly-OH (10 eq., 0.12 mmol per part) in DMF
10 overnight.

       The encoding procedure: Cleavage of Lys(Tfa)-
protecting group (identical for all 36 parts): The
resin was washed with water and treated with 20%
piperidine/H_2O (two times, 10+30 min). The resins were
15 then washed with water and DMF. Subsequently, the tag
for all sequences was synthesized by Fmoc-strategy
(DIC/HOBt activation, 10 eq, 0.14 mmol per part)
without cleavage of the N-terminal Fmoc-protecting
group of the tag. Coding sequences for the tag are
20 shown in Figure 8. All 36 portions were then
collected, mixed and divided into two portions. To
one half only, Boc-Gly was coupled after the Boc-group
deprotection in the main chain. Both portions were
mixed, the Boc-protecting group was removed and the
25 resin divided into 13 portions. Fmoc-Tyr(Bu')-OH,
Fmoc-Trp-OH, Fmoc-Lys(Boc)-OH, Fmoc-Thr(Bu')-OH, Fmoc-
His(Trt)-OH, Fmoc-Pro-OH, Fmoc-Phe-OH, Fmoc-Arg(Pmc)-
OH, Fmoc-Asn-OH, Fmoc-Ser(Bu')-OH, Fmoc-Glu(OBu')-OH,
Fmoc-Asp(OBu')-OH and Fmoc-Gln-OH (10 eq, 0.38 mmol)
30 were coupled by DIC/HOBt activation to those 13
portions. Side chain deprotection: Boc- and Bu'-side
chain protection groups were cleaved by
TFA/DCM/anisole (50:50:2) (15 min exposure), the Trt-
group was cleaved by the same mixture with addition of
35 1 drop of 'Pr$_3$SiH for 15 min. The Pmc-group was

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cleaved by mixture K for 1 hour. After washing and
neutralization steps (DCM, 7% DIEA/DCM, DMF), all 13
portions were collected and the N-terminal Fmoc-group
was cleaved. The library was then washed with DMF and
5   transferred into 0.1% aqueous HCl.

9.1.9 SCREENING PROTOCOL OF THE LIBRARY

The peptide library was screened according to the
published procedure (Lam and Lebl, 1992, Immunomethods
10 1:11-15). The peptide beads were first mixed with
double-distilled water to remove the DMF. After
extensive washing with PBS (137 mM NaCl, 2.7 mM KCl,
4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2), the beads were
coated with 0.05% gelatin (w/v) to block any
15 nonspecific binding. The beads were then incubated
with 60 pM biotinylated anti-β-endorphin antibody
(clone 3-E 7, Boehringer Mannheim) in 2 x
PBS/Tween/gelatin overnight. After thorough washing,
streptavidin-alkaline phosphatase was added. One hour
20 later, the beads were washed, the substrate was added,
and color development proceeded as described above.
The colored beads were then physically isolated and
subjected to sequencing.

25   ### 9.2 RESULTS AND DISCUSSION

An alternative non-peptidic scaffolding is based
on the use of Kemp's triacid (Kemp and Petrakis, 1981,
J. Org. Chem. 46:5140-5143) (Scheme XV).

30

35

SCHEME XV

- 137 -
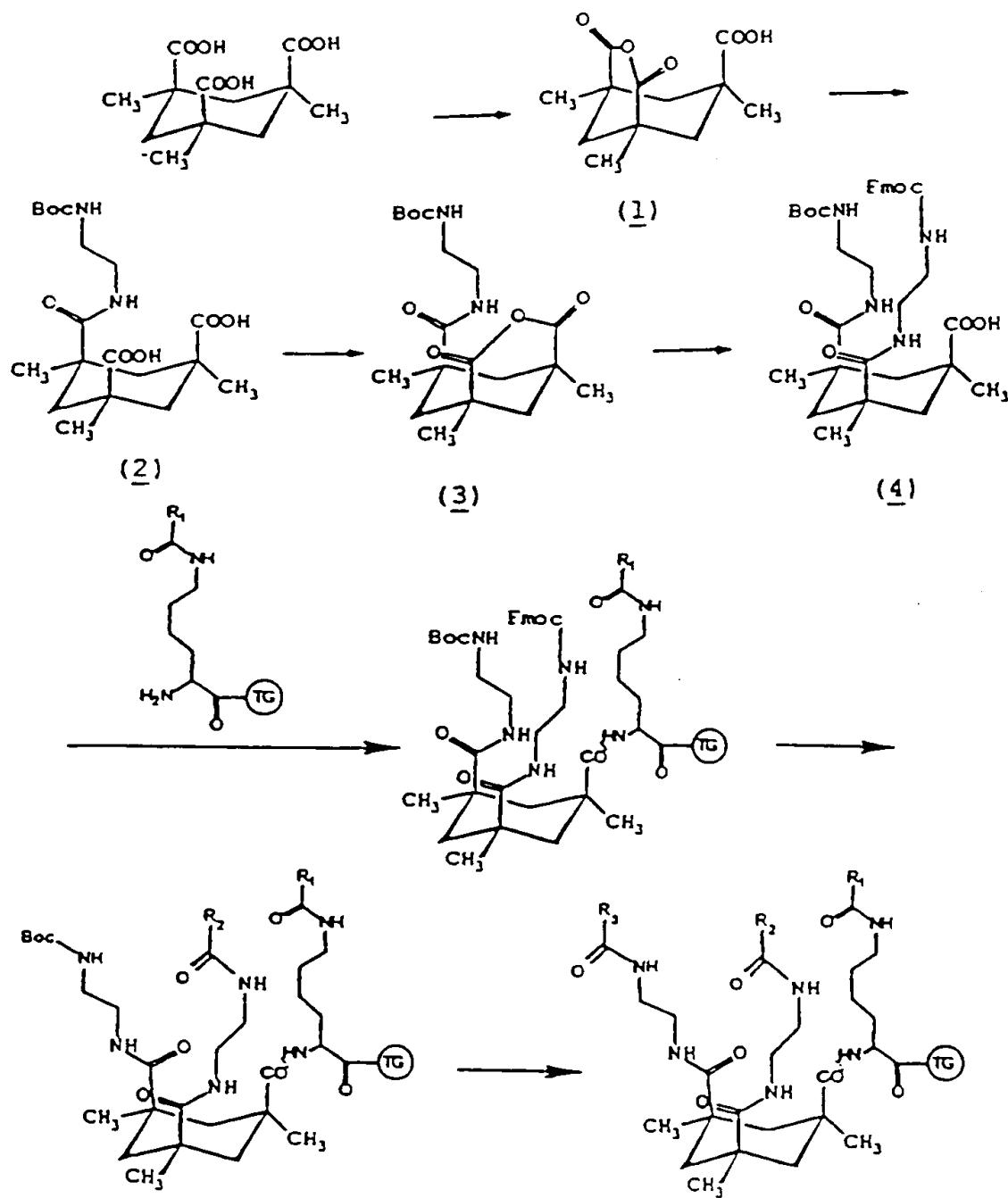
     In this structure the three carboxyl groups are constrained to the triaxial conformation. Anhydride acid 1 was prepared by dehydration procedure (Askew et al., 1989, J. Am. Chem. Soc. 111:1082-1090) using a

5 Dean-Stark trap under nitrogen. This acid anhydride was opened by nucleophilic attack by mono tert-butyloxycarbonylethylenediamine yielding amide diacid 2. The same method for dehydration is not applicable for this amide diacid because of instability of Boc

10 protecting group under these conditions. A mild alternative for preparation of the amide anhydride 3, peculiar to peptide chemistry was the use of the common dicyclohexylcarbodiimide procedure in methylene chloride. The amide anhydride 3 was then opened by

15 fluorenylmethyloxycarbonyl-ethylenediamine to the corresponding diamide acid 4. Mono protected ethylenediamines have been prepared starting from Boc-ethylenediamine which was prepared by bocylation of ethylenediamine using tert-butyl dicarbonate (Krapcho

20 et al., 1990, Synthesis Commun. 20:2559-2564). Mono Boc-ethylenediamine was used as such and it served also as starting compound for preparation of mono fluorenylmethyloxycarbonyl-ethylenediamine trifluoroacetate via N-Boc-N'-Fmoc-ethylenediamine.

25   Diamide acid 4 can be used either for the synthesis of scaffolding bearing the third chain containing orthogonal protecting group (Ddz, Alloc) and carboxyl function or as scaffold itself providing that the third randomization is done separately. The

30 use of 1 as a scaffolding was chosen to synthesize the totaly nonpeptidic library. The first randomization was done on lysine side chain although any trifunctional amino acid can be used for this purpose, and the second and the third randomization was

35 performed on the scaffolding. Using this

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conformationally constrained scaffolding a non-peptide
library randomized with 20 different carboxylic acid
was built.

     A scaffolding mapping a larger conformational
5   space is a simple branched attachment constructed by
consecutive coupling of diamino carboxylic acids.
Various types of the scaffolding mapping extensive
space are flexible cyclic or branched scaffoldings.
The principles of these libraries are illustrated
10 generally in Figure 4. Scheme XVI shows a specific
example of such a library:

15

20

25

30

35

SCHEME XVI

Tfa⌐
Fmoc — βAla — Lys — βAla — Lys — Lys-TG
                        Npys⌐   Ddz⌐

Tfa⌐
Fmoc — βAla — Lys — Lys-TG
                   Ddz⌐

Tfa⌐
R₁ — βAla — Lys — βAla — Lys — Lys-TG
              R₂⌐   Fmoc-AA2-AA1⌐

Tfa⌐
R₁ — βAla — Lys — βAla — Lys — Lys-TG
              Npys⌐   Fmoc-AA1⌐

Npys⌐
Fmoc — βAla — Lys

R₁ — βAla — Lys — βAla — Lys — Lys-TG
              R₂⌐   Ddz-Phe-AA2-AA1⌐

Tfa⌐   R₁ — βAla — Lys — βAla — Lys — Lys-TG
R₁ — βAla — Lys — βAla — Lys — Lys-TG
         R₂⌐   Ddz-Phe-AA2-AA1⌐

R₄⌐
R₃ — βAla — Lys

Npys⌐
R₃ — βAla — Lys

R₁ — βAla — Lys — βAla — Lys — Lys-TG
         R₂⌐ Fmoc-AA3-Phe-AA2-AA1⌐

R₁ — βAla — Lys — βAla — Lys — Lys-TG
         R₂⌐   AA4-AA3-Phe-AA2-AA1⌐

The synthesis of this scaffolding required the use of
four independent (orthogonal) protecting groups. We
have tested the use of trifluoroacetyl group
introduced into peptide chemistry in the fifties
(Schallenbert and Calvin, 1955, J. Am. Chem. Soc.
77:2779), but that was not used due to the harsh
conditions required for its deprotection, as well as
due to its ineffectiveness in protection against
racemization when used as α-amino group protection.
We have found that this group is not cleaved during

Fmoc deprotection using 50% piperidine in
dimethylformamide, but it is completely cleaved by 1 -
2 h exposure to piperidine solution (20%) in water,
which, however, also cleaves Fmoc group. The strategy
5 used in the construction of this library is clear from
Scheme XV.
        Non-amino acid subunits can be combined with
standard amino acids. We have shown that this
approach can yield reasonable binding structures by
10 constructing the minilibrary of 936 members, having
selected amino acids randomized in position 1, one or
two glycines in position 2 and 3 and a set of aromatic
amines coupled to the β-carboxyl group of aspartic
acid or side chain modified iminodicarboxylic acid, or
15 aromatic acids coupled to the side chain of
diaminobutyric acid in position 4, or benzylhalides
coupled to the side chain of sulfur containing amino
acids (cysteine and penicillamine). The structure of
the library is shown in Scheme XVII:
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SCHEME XVII

$$X_1\text{-}(G)_{1\text{-}2}\text{-}X_2\text{-}G\text{-}\beta G\beta G\text{-}K\text{-}\boxed{TG}$$

$$(X_2)^1\text{-}(X_2)^2$$
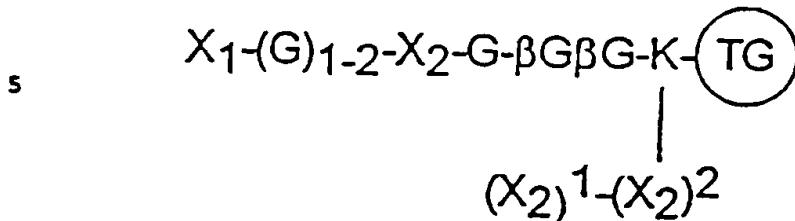
- X_1 = Tyr, Trp, Lys, Thr, His, Pro, Phe, Arg, Asn, Ser, Glu, Asp, Gln
- X_2 = Building blocks 1-36
- $(X_2)^{1\text{-}2}$ = Coding sequence

Total number of permutations: 936

Position 4, which contains the non-amino acid subunit, may create problems during the sequencing and therefore this position has been encoded. Since more than 20 building blocks were used in the randomization, a doublet amino acid coding strategy was used (Figure 8). To avoid complications in structural determination, the amino acids used for coding do not overlap with the set used for the randomization of the position 1 and amino acid in position 2. Using a doublet codon of 6 amino acids, up to 36 different building blocks could be encoded.

This minilibrary was screened against a model system, anti-β-endorphin monoclonal antibody. Positively reacting beads were subjected to three cycles of Edman degradation, and the interacting structures, deduced from the obtained data are given

in Scheme XVII. The structure of the natural ligand
for the anti-β-endorphin monoclonal antibody is also
shown in Scheme XVIII:

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SCHEME XVIII

Found in library:

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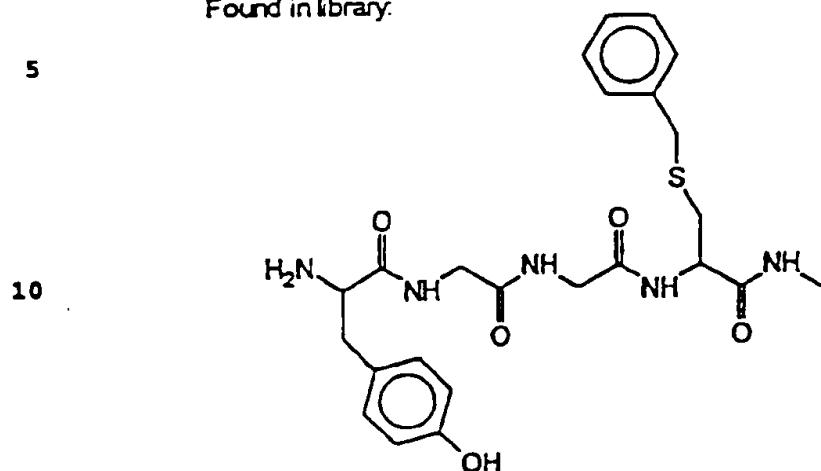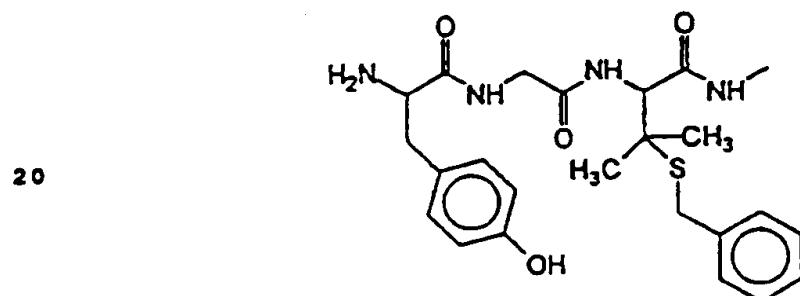
25

Natural ligand:

30
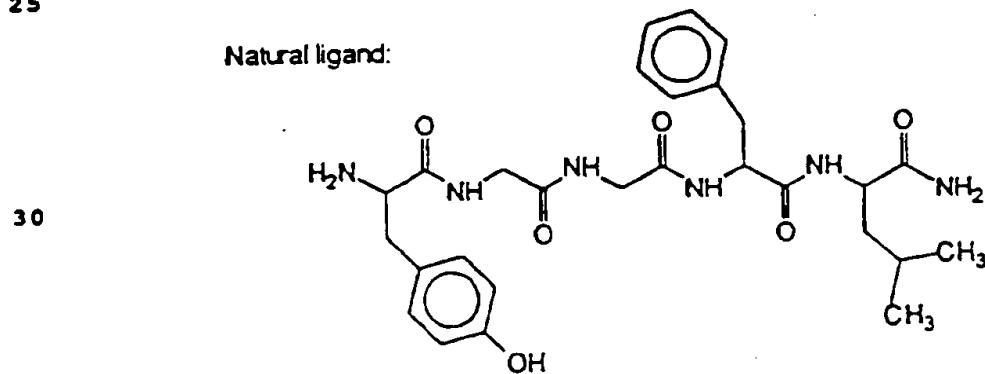
35

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Compounds that were selected on the basis of binding
with the anti-β-endorphin monoclonal antibody were
synthesized bound to the beads and in the free form
and their binding affinities were determined. Bead
5 bound sequences have shown specific binding
(competable by leucine enkephalin). As can be seen
(Scheme XVIII), the binding to the antibody requires
two aromatic groups in the appropriate distance. The
structure connecting those two aromatic groups is
10 nevertheless very important for the binding affinity.

      10.   EXAMPLE: SELECTIVE ACTIVATION OF SURFACE
              FUNCTIONAL GROUPS ON A RESIN BEAD

        The present example describes preparation of a
15 solid phase support particle having a "surface" of the
particle physically separate from the "inside" of the
carrier, and synthesizing the screening structure on
the surface and the coding molecule inside of the
bead. The surface in this sense should be understood
20 as the portion of the bead accessible to the
macromolecular acceptor molecule. The available
surface of the bead corresponds approximately to the
calculated surface area based on the bead dimensions
for an acceptor molecule of extremely high molecular
25 weight. Alternatively, the surface area can be
determined by various methods utilizing penetration
into a material (including inner surface of all pores
in the polymeric bead) for acceptor molecules with low
molecular weight. Understandably, acceptor molecules
30 that freely penetrate the polymeric network will not
recognize any difference between surface and inside of
the polymeric particle. The available surface of the
particle also include a dynamic component.

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10.1   MATERIALS AND METHODS

10.1.1   REMOVAL OF THE SURFACE CONTENT OF
           THE PEPTIDE FROM SOLID PHASE BEAD

Model peptides (YGGFL, LHPQF, LHPQFYG) were
synthesized on TentaGel AM (Rapp Polymere, Tubingen,
Germany, 0.21mmol/g) having the linker β-Ala-Gly-β-
Ala-Gly attached to it. Synthesis was performed by
standard solid phase technique utilizing Fmoc
protected amino acids and diisopropylcarbodiimide in
the presence of N-hydroxybenzotriazole as a coupling
reagent. Peptides were deprotected in two steps using
trifluoroacetic acid with scavengers (ethanedithiol,
water, and thioanisole), and piperidine (20%) in
dimethylformamide. Beads were carefully washed and
transferred to 0.1 M ammonium carbonate buffer of pH
7.7. Chymotrypsin (1mg) was added and suspension was
shaken at 37°C for 20 hours. The same treatment was
repeated twice for 4 hours. Sequencing of randomly
selected beads from every group have shown that the
peptide content on the beads did not change
significantly.

10.1.2   SYNTHESIS OF DIFFERENT PEPTIDES ON
           THE SURFACE AND INSIDE OF THE POLYMERIC
           BEAD

TentaGel AM resin (0.21mmol/g, 1g) was modified
by condensation of Boc-Phe. Resin was washed and
transferred to the 0.1M ammonium carbonate buffer pH
7.7. Chymotrypsin treatment was performed in the same
way as described above. After careful washing by the
same buffer and water, resin was washed with
dimethylformamide and the standard solid phase
synthetic scheme using Fmoc protecting groups and
diisopropylcarbodiimide and N-hydroxybenzotriazole as
coupling reagent was used for the synthesis of YGGFL
sequence. After the coupling of Fmoc-Tyr(But) in the

last coupling step, Fmoc group was not removed and the
resin was treated with 50% trifluoroacetic acid in
dichloromethane. In the next synthetic steps Boc
protected amino acids were used. Coupling was
5 performed using the same reagent as above, the Boc
group was removed after each step by 50%
trifluoroacetic acid and the protonated amino group
was made available for the coupling by treatment with
diisopropylethylamine solution in dimethylformamide
10 (5%). Peptides were deprotected in two steps using
trifluoroacetic acid with scavengers (ethanedithiol,
water, and thioanisole), and piperidine (20%) in
dimethylformamide. Beads were carefully washed and
prepared for staining with anti-β-endorphin as
15 described previously. Staining of the beads was
indistinguishable from staining of the beads
containing only YGGFL sequence.

       10.2   RESULTS AND DISCUSSION
20   An enzyme has been used to selectively cleave a
peptide from the surface of a bead. The test
sequences YGGFL and LHPQFYG were prepared and
incubated with chymotrypsin and the reactivity of the
beads was tested.
25   Treated beads were tested for binding to anti-β-
endorphin and streptavidin. Results are given in the
Table 5.

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Table 5

Binding to the Beads After Treatment With Chymotrypsin				

Peptide on Bead	Binding to			
	Antibody		Streptavidin	
	No CT	+ CT	No CT	+ CT
	3+	-	-	-
YGGFL				
LHPQF	-	-	3+	2+
LHPQFYG	-	-	3+	-

CT = chymotrypsin

Beads with YGGFL completely lost binding activity
to anti-β-endorphin antibody (Table 5). Beads
containing LHPQF-YG also completely lost activity with
streptavidin. However, the decrease in the total
amount of LHPQF-YG or YGGFL was minimal, indicating no
effect on the interior of the bead. In this case,
since LHFQF was a poor substrate for chymotrypsin, the
YG linker was used. Edman degradation showed that the
peptide content on the beads did not change
significantly.

Based on these preliminary results, chymotrypsin
was used as a selective deprotecting reagent. A
simple substrate for chymotrypsin, Boc-Phe, was
synthesized on all available amino groups of the solid
carrier. Incubation of this modified resin under
various conditions led to the release of approximately
the same amount of Boc-Phe: 1.1%. The deprotected
amino group was used for the synthesis of YGGFL using
Fmoc strategy. Synthesis was followed by quantitative
measurement of Fmoc release, and the readings

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confirmed that approximately 1% of available amino groups were used for the synthesis. The Boc protecting group from the remaining amino groups on each bead was cleaved by TFA and the sequence LHPQF

5   was assembled using Boc synthesis. All protecting groups were removed and the testing revealed positive reaction with both anti-β-endorphin and streptavidin.

Randomly selected beads were submitted for Edman degradation and the following results were obtained

10 (values in pmoles): 1st cycle: L 114, Y <1; 2nd cycle: H 86, G <1; 3rd cycle: P 94, G <1; 4th cycle: Q 78, F 4 (preview); 5th cycle: F 73, L <1. Edman degradation analysis show the sequence LHPQF at the level of 100-120 pmoles per cycle, and negligible content of amino

15 acids expected for YGGFL sequence. This means that even though YGGFL is not present in the bulk of the bead, it is available on the surface of the bead for the interaction with the relevant acceptor.

LHPQF is also still available for interaction

20 with streptavidin. This is probably due to the lower molecular weight of streptavidin, allowing it to penetrate inside of the polymeric bead.

10.3   DISCUSSION

25 The present Example clearly demonstrates that an enzyme can selectively deprotect the surface, but not the interior, of a resin bead. In this case, chymotrypsin cleavage of the sequence LHPQFYG proceeded efficiently. Binding of streptavidin, which

30 is specific for LHPQF, was completely abrogated.

Subsequent experiments with a Boc-Phe substrate derivatized beads showed that two different peptides can be synthesized on a bead, with one peptide segregated primarily to the surface and the other

35 peptide segregated primarily to the inside.

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11.   EXAMPLE: PREFERENTIAL BLOCKING OF THE FREE
      AMINO GROUPS ON THE SURFACE OF A TENTAGEL
      BEAD

          The present Example demonstrates the
feasability of blocking the surface of a resin bead
while leaving the functional groups in the interior of
the bead free for reaction and synthesis of a
compound, for example, a coding molecule.

11.1   MATERIALS AND METHODS

          TentaGel amide AM resin (0.3 meq/gram
substitution) was hydrated in H_2O for several hours,
and washed with 0.1 M 2-[N-morpholino]ethanesulfonic
acid (MES) buffer, pH 5.7. A varying amount (0 to 10
mg) of polyglutamic acid (Mr 30,000, Sigma Chemical)
was added to each of the 0.2 ml of settled bead.
Sixty mg of 1-ethyl-3-(3-dimethylamino-propyl)
carbodiimide-HCl (EDC) was then added to each tube.
The reaction mixtures were rocked gently overnight.
The beads were then washed into double distilled (dd)
H_2O and 0.24 mmol ethanolamine plus 60 mg EDC in MES
buffer were added to each sample. After 4 hours of
gentle shaking, the resin was washed extensively with
dd H_2O. The resin was then lyophilized to dryness.
The treated resin was swollen in dimethylformamide
(DMF). Using Fmoc chemistry, the peptide, YGGFLGGG
was synthesized on each of the treated resin samples
using standard techniques. The N-terminal Fmoc group
and the side chain protected groups were removed with
piperidine and trifluoracetic acid as described (Lam
et al., 1991, Nature 354:82-84). After neutralization
with diisopropylethylamine (DIEA), the resin beads
were washed extensively and the beads prepared for
staining with anti-β-endorphin as described in Section
10, supra.

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11.2   RESULTS AND DISCUSSION

      The ability to block surface functional
groups on a resin bead with polyglutamic acid was
directly proportional to the amount of polyglutamic
5 acid used (Table 6). Significantly, the polyglutamic
acid-blocked resin stained only weakly, suggesting
that most of the surface amino groups were blocked
prior to the synthesis of the YGGFLGGG peptide. More
importantly, however, was that the amount of peptide
10 in both the blocked and unblocked resin was about the
same (Table 7) indicating that the polyglutamic acid
block occurred only on the surface of the bead, and
that the free amino groups inside the beads remained
intact for subsequent peptide synthesis.

15

Table 6

Semi-Quantitative
Staining of Peptide-Beads

Extent of Blocking: Polyglutamic Acid, mg. per 0.2 ml Resin	Labeling With Biotinyl-Anti-β Endorphin Followed by Streptavidin-AP	Labeling With Streptavidin-AP Alone
0	3+	−
0.016	3+	−
0.08	2+	−
0.04	1+	−
2.0	1+	−
10	trace	−

Table 7
Micro Sequencing
pmole amino acid (10 beads/experiment)*

		Y	G	G	F	L	G	G	G
Polyglutamic Acid (10 mg)	I	609,	615,	692,	665,	579,	657,	675,	655
	II	996,	885,	839,	772,	696,	522,	550,	506
Without Blocking	I	876,	752,	752,	774,	680,	733,	707,	717
	II	519,	534,	332,	559,	471,	540,	550,	335

Additional experiments have involved reversibly coupling polymer onto which Boc-Glu was attached via its α-carboxly group. After the coupling of this modified polymer onto the bead surface and appropriate modification of "inside" amino group, the Boc group from glutamic acid was cleaved. The beads were then treated with one cycle of Edman degradation, which regenerated amino groups that had been protected by the glutamic acid polymeric reagent. Different peptides were synthesized on separate parts of the bead, i.e., on the surface and inside.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

12. EXAMPLE: SUBUNITS SUITABLE FOR THE
CONSTRUCTION OF NONPEPTIDIC LIBRARIES

The construction of nonpeptidic libraries
requires the selection of subunits suitable for
5   inclusion into solid phase synthesis. The present
example concerns the selection of subunits suited to
three different chemical methodologies.

12.1   ACYLATION OF PRIMARY AMINO GROUP

10   Experimental Protocol: One mmol of acid was
dissolved in 2 ml of DMF, 0.5 mmol of DIC added (1
mmol in the case of dicarboxylic acids) and reacted
with approximtely 100 mg of Trp-RAM-TG overnight. The
completion of the reaction was assessed by Kaiser
15   test. The resin was then washed with DMF, DCM, dried.
The product was cleaved by one hour's exposure to 1 ml
of 95% TFA, 5% water. The reaction mixture was ten
diluted with with 5 ml water and analyzed by HPLC and
MS.

20   The requirements for acceptance were that the
products have the expected M.W. and the content of the
main product in a crude mixture is more than 80% of
the total area under the curve of the HPLC
chromatogram and that the major product have the
25   expected molecular weight. Table 8 displays the
results of the tests of 33 acids. Of the 33, 7 were
rejected as unsuitable for use. A further two
compounds, 4-hydroxybenzoic acid and 4-
hydroxyphenylacetic acid, were incompatible with mono-
30   synthesis using tryptophan but were found usable in
different model experiments using the N^{α}-amino group of
lysine(Fmoc).

35

TABLE 8 - Acylation of Primary Amino Group

Acid	Solubility	Kaiser	HPLC	MW	MS	Selected
γ-Guanidinobutyric	heat	-	OK	330	OK	Yes
Succinamic	OK	-	OK	302	OK	Yes
1-Naphtylacetic	OK	-	OK	371	OK	Yes
Diphenylacetic	OK	-	2 peaks	397	OK	Yes
Biphenylacetic	OK	-	OK	397	OK	Yes
Pentafluorophenylacetic	OK	-	OK	411	OK	Yes
4-Trifluoromethylbenzoic	OK	-	OK	375	OK	Yes
4-Hydroxybenzoic	OK	(+)		323	not OK	Yes
4-Hydroxyphenylacetic	OK	-	OK	337	not OK	Yes
3-Nitrophenylacetic	OK	+	2 peaks	366	OK	Yes
4-Methoxybenzoic	OK	-	2 peaks	337	OK	No
2-Nitro-4,5-dimethoxybenzoic	OK	-	OK	412	OK	Yes
3-(3,4,5-trimethoxyphenyl)propionic	OK	++	OK	425	OK	Yes
4-Guanidinobenzoic	OK	++	2 peaks	364	OK	Yes
4-Dimethylaminobenzoic	OK	+	OK	350	OK	Yes
4-(Pyrrole-1-yl)benzoic	OK	-	not OK	372	not OK	No
4(3-Methyl-5-oxo-2-pyrazolin-1-yl)benzoic	heat	red	OK	403	OK	Yes
2-Pyrrolecarboxylic	OK	(+)	OK	296	not OK	No
1-Methyl-2-pyrrolecarboxylic	HOBt, heat	++	not OK	310		No

TABLE 8 - Acylation of Primary Amino Group - (cont'd.)

Acid	Solubility	Kaiser	HPLC	MW	MS	Selected
1,4-Dimethyl-2,3-pyrroledicarboxylic	OK	(+)	OK	382	OK	Yes
2-Methyl-4-nitro-1-imidazolepropionic	OK	-	OK	384	OK	Yes
2-Amino-1-imidazoleacetic	HOBt, heat	++	not OK	328		Yes
2 Pyrazolecarboxylic	OK	-	not OK	297		No
3-amino-1,2,4-triazole-5-carboxylic				313		Yes
4-Imidazoleacetic	heat	++	OK	311	OK	Yes
i-nicotinic	HOBt, heat*			308		No
2-Hydroxy-i-nicotinic	OK	(+)	not OK	324		No
2,3-Pyridinedicarboxylic	OK	-	2 peaks	352	OK	Yes
2-Pyrazinecarboxylic	heat	-	OK	309	OK	Yes
2,3-Pyrazinedicarboxylic	OK	-	OK	353	OK	Yes
1-Methylindole-2-carboxylic	OK	-	OK	360	OK	Yes
2-Methyl-3-indoleacetic	OK	-	OK	374	OK	Yes
Indole-4-carboxylic	OK	-	OK	346	OK	Yes

*   precipitated after cooling

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12.2 NUCLEOPHILIC DISPLACEMENT OF HALOGEN BY AMINES

Experimental Protocol: Bromoacetic acid (5x molar excess) was coupled via symmetrical anhydrides to TG130 for 10 min, recoupled for 10 min.

5 using the same method. The test amine was coupled using a 1M solution in DMSO, overnight. After suitable washing Fmoc-Gly was coupled (HOBt, DIC) for 1 hour, the Fmoc group removed, and Fmoc release calculated and compared to a theoretical release 45 μmol/ml. In

10 a second set of experiments, the purity and identity of products were tested by the synthesis of the model compounds R-NH-CH$_2$-CO-Gly-Trp-RAM-TG, synthesized by a similar protocol and cleaved from the resin with 1 ml of 95% TFA, 5% water for 1 hour, followed by dilution

15 with about 5 ml of water to perform analytical HPLC and MS.

A total of 33 amines were tested of which 21 were found to be acceptable for incorporation into solid phase synthesis. The results of these studies are given below.

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Amine	MW	Fmoc	HPLC	MS	Selected
Ethylamine	45.08	31	OK	OK	Yes
i-Propylamine	59.11	27	OK	OK	Yes
Butylamine	73.14	41	OK	OK	Yes
i-Butylamine	73.14	24	OK	OK	Yes
t-Butylamine	73.14	4	*	*	No
Cyclopentylamine	85.15	28	OK	OK	Yes
Cyclohexylamine	99.18	24	OK	OK	Yes
2-Amino-norbornane	111.19	35	OK	OK	No
Ethanolamine	61.08	31	OK	OK	Yes
3-Aminopropanol	75.11	40	OK	OK	Yes
1-Amino-2-propanol	75.11	43	OK	OK	Yes
2-Methoxyethylamine	75.11	26	OK	OK	Yes
Tetrahydrofurfurylamine	101.15	27	OK	OK	No
β-Ala-OtBu	181.6	22	OK	OK	Yes
Ethylendiamine(Boc)	120	29	OK	OK	Yes
2-(2-Aminoethyl)-1-methylpyrrolidine	128.1	35	OK	OK	Yes
1-Aminopiperidine	100.17	2.2	*	*	No

Table 9 - Nucleophilic Displacement of Halogen by Amines

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Amine	MW	Fmoc	HPLC	MS	Selected
1-Amino-4-methylpiperazine	115.0	20	Not OK	Not OK	No
4-Aminomorpholine	101.14	3	*	*	No
Benzhydrylamine	183.25	12.5	Not OK	Not OK	No
Naphthalene-methylamine	157.22	24.7	OK	OK	Yes
2-Aminoindan	169.96	0	*	*	No
4-(Trifluoromethyl)-benzylamine	175.16	29	OK	OK	Yes
2-Amino-1-phenyl-ethanol	137.18	29	OK	OK	Yes
Tyramine	137.18	27	OK	OK	Yes
4-Methoxy-benzylamine	137.18	34	OK	OK	Yes
3,5-Dimethoxy-benzylamine	167.21	31	OK	OK	Yes
4-(Dimethylamino)-benzylamine	150.22	not soluble in DMSO			No
2,4-Dinitrophenyl-hydrazine	198.0	5	*	*	No
Aniline	93.18	1.2	*	*	No
p-Toluidine	143.0	7.0	*	*	No
p-Anisidine	123.15	2.8	*	*	No

Table 9 - Nucleophilic Displacement of Halogen by Amines (cont'd.)

* Amines, with low Fmoc release. Experiment was repeated and if low Fmoc release occurred again, the amine was excluded from further experiments.

12.3   REDUCTIVE ALKYLATION

12.3.1 GENERALIZED METHODS

Experimental Protocol: A 20 molar excess of the
test aldehydes were added to about 100 mg of (Exp. A)
5   H-Gly-RAM-TG or (Exp. B) H-βAla-Gly-Trp-RAM-TG swollen
in 1 ml MeOH-DCM-AcOH 40:10:1, and the reaction was
allowed to proceed overnight. The resin was washed
with 1% AcOH in DMF and then reexposed to a 20 molar
excess of the test aldeyde overnight. Thereafter the
10 resin was washed with MeOH-DCM-AcOH 40:10:1, and to 1
ml of this mixture a 20 molar excess of $NaBH_3CN$ was
added and allowed to react overnight. The resin was
then washed with DMF/1% AcOH, and a second 20 molar
excess of $NaBH_3CN$ was added for an overnight reaction.
15 After washing, all samples were acylated with Fmoc-
Gly. In the Exp. A, after removing the Fmoc, the
product was acylated with Fmoc-Trp. The resin was
then treated to remove the Fmoc, washed with DMF, DCM,
dried, the product was then cleaved with 1 ml of 95%
20 TFA, 5% water for 1 hour. After Dilution with about 5
ml water, analytical HPLC and MS were performed.

A total of 31 test aldehydes were tested to
determine their suitablility for inclusion in the
solid phase synthesis. The results, given in Table 10
25 indicate that 19 of these were acceptable.

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Table 10 - Reductive Alkylation					
Aldehyde	Kaiser	HPLC	MW	MS	Selected
Experiment "A"					
Butyraldehyde	brown	not OK			No
2-Methylbutyraldehyde	light blue	OK	387	OK	Yes
2-Ethylbutyraldehye	brown	OK	401	OK	Yes
Trimethylacetaldehyde	brown	OK	387	OK	Yes
i-Valeraldehye	brown	not OK			No
2-Methylvaleraldehyde	light blue	OK	401	OK	Yes
3-Methylthiopropionaldehyde	light blue	not OK			No
Cyclohexanecarboxaldehyde	light blue	OK	413	OK	Yes
5-Noorbornene-2-carboxaldehyde	brown	not OK			No
Benzaldehyde	brown	OK	407	OK	Yes
4-Noitrobenzaldehyde	brown	OK	452	OK	Yes
4-Hydroxybenzaldehyde	light blue	OK	423	OK	Yes
Hydrocinnamaldehyde	brown	not OK			No
Vanillin	brown	OK	453	OK	Yes
2-Thiophenecarboxaldehyde	light blue	OK	413	OK	Yes
Imidazole-2-carboxaldehyde	brown	no OK			No
Pyridine-4-carboxaldehyde	brown	OK	408	OK	Yes
α,α,α-Trifluoro-o-tolualdehyde	brown	OK	475	OK	Yes

TABLE 10 - Reductive Alkylation (cont'd.)					
Aldehyde	Kaiser	HPLC	MW	MS	Selected
Experiment "B"					
4-Dimethylaminobenzaldehyde	brown	not OK			No
4-Methoxybenzaldehyde	brown	OK	507	OK	Yes
2,4,6-Trimethoxybenzaldehyde	brown	not OK			No
Indole-3-carboxaldehyde	brown	not OK			No
1-Acetylindole-2-caboxaldehyde	brown	OK	559	nd	Yes
4-Carboxybenzaldehyde	light blue	OK	522	not OK	No
β-naphtaldehyde	brown	OK	528	OK	Yes
Phenylacetaldehyde	brown	not OK			No
4-Phenylbenzaldehyde	brown	OK	554	OK	Yes
3-Phenoxybenzaldehyde	brown	OK	570	OK	Yes
Pyrrole-2-carboxaldehyde	brown	not OK			No
2-Hydroxybenzaldehyde	brown	OK	494	OK	Yes
Quinoline-4-carboxaldehyde	blue	not OK			No

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12.3.2   SPECIFIC METHODS

The following gives procedures specifically suited for the subunit to be added.

Sequence β-Ala-Gly-Trp was assembled on RAM-TG
5 (subst. 0.2 mmol/g). The terminal amino group was then alkylated using reductive amination procedure with a set of aliphatic, aromatic, and heterocyclic aldehydes. N-alkylated peptides were split of the resin using 95% TFA - 5% H_2O and the purity and the
10 correct molecular weight of the resulting compounds were confirmed using HPLC and mass spectroscopy.

Procedure 1
solvent mixture 1   dichloromethane-methanol-acetic
acid 80:20:1
15 solvent mixture 2   dimethylformamide-acetic acid
100:1

Schiff base formation: To a 50 mg of H-β-Ala-Gly-
Trp-TentaGel S RAM washed 3x with solvent mixture 1
were added 200μl of this mixture and 0.2 mmol of
aldehyde . Resin was shaken for 2 hours, then washed
3x with solvent mixture 2. Another 0.2mmol of aldehyde
20 together with 200μl of solvent mixture 2 were added
and after 2 hours shaking resin was washed 3x with
solvent mix.2 and 3x with solvent mix. 1.

Schiff base reduction: To a washed peptide-resin
200 μl of solvent mix. 1 and 200 μl of 1M solution of
$NaBH_3CN$ in dimethylformamide were added. Resin was
25 shaken for two 2 hours, then washed with solvent mix.
2 and reduction repeated in this mixture with 200 μl
of 1 M solution of $NaBH_3CN$ in DMF for another 2 hours.
Resin was then washed with DMF. DCM, dried, and
peptide was cleaved using TFA-5%H_2O.

30 Protocol 1
wash 3x with solvent mix. 1
add 200 μl of solvent mix. 1
add 0.2 mmol of aldehyde
shake for 2 hrs
wash 3x with solvent mix. 2
add 200 μl of solvent mix. 2
35 add 0.2 mmol of aldehyde
shake for 2 hrs
wash 3x with solvent mix. 2
wash 3x with solvent mix. 1

add 200 μl of solvent mix. 1
add 200 μl of 1M NaBH$_3$CN in DMF
shake for 2 hrs
wash 3x with solvent mix. 2
add 200 μl of solvent mix. 2
add 200 μl of 1M NaBH$_3$CN in DMF
5   shake for 2 hrs

Procedure 2
Schiff base formation follows procedure A.
Schiff base reduction follows procedure A with the
difference that during the both reduction steps 0.01
10   mmol of aldehyde was added together with reduction
reagent.

Protocol 2
wash 3x with solvent mix. 1
add 200 μl of solvent mix. 1
add 0.2 mmol of aldehyde
shake for 2 hrs
15   wash 3x with solvent mix. 2
add 200 μl of solvent mix. 2
add 0.2 mmol of aldehyde
shake for 2 hrs
wash 3x with solvent mix. 2
wash 3x with solvent mix. 1
add 200 μl of solvent mix. 1
20   add 200 μl of 1M NaBH$_3$CN in DMF
add 0.01 mmol of aldehyde
shake for 2 hrs
wash 3x with solvent mix. 2
add 200 μl of solvent mix. 2
add 200 μl of 1M NaBH$_3$CN in DMF
add 0.01 mmol of aldehyde
shake for 2 hrs
25

Procedure 3 (one-pot reaction)
To a 50 mg of peptide-resin washed with solvent
mixture 2 was added 200 μl of this mixture and 0.05
30   mmol of aldehyde. After 1 hour of shaking 50μl of 1M
solution of NaBH$_3$ in DMF was added and resin was
allowed to shake for 2 hours. Then another 50 μl of
reductive reagent was added and after 2 hours shaking
the third portion (50 μl) of 1M solution of NaBH$_3$CN in
DMF was added. Resin was shaken overnight and then
worked up as above.

35   Protocol 3
wash 3x with solvent mix. 2
add 200 μl of solvent mix. 2
add 0.05 mmol of aldehyde

shake for 1 hr
add 50 μl of 1M NaBH$_3$CN in DMF
shake for 2 hrs
add 50 μl of 1M NaBH$_3$CN in DMF
shake for 2 hrs
add 50 μl of 1M NaBH$_3$CN
5   shake overnight

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TABLE 11 - Reductive Amination		
Aldehyde (M.W.)	Prtcol.	MS (MH+)
benzaldehyde (421.2)	1	OK (422.2)
2-hydroxybenzaldehyde (437.2)	1	OK (438.2)
4-hydroxybenzaldehyde (437.2)	1	OK (438.2)
4-hydroxybenzaldehyde (451.2)	3	OK (438.2)
3-methyoxy-4-hydroxybenzaldehyde (467.2)	1	OK (468.2)
4-nitrobenzaldehyde (466.3)	1	OK 467.3)
2-naphtaldehyde (471.3)	1	OK (472.3)
3-phenoxybenzaldehyde (513.3)	1	OK (514.3)
4-phenylbenzaldehyde (497.3)	1	OK (498.3)
2-tolualdehyde (435.2)	1	OK (436.2)
2-trifluoromethyl-benzaldehyde (489.3)	2	OK (490.3)
1,3,5-trimethoxybenzaldehyde (511.3)	1	OK (512.3)
cyclohexanecarboxaldehyde (427.2)	2	OK (428.2)
2-ethylbutyraldehyde (415.2)	2	OK (416.2)
2-methylbutyraldehyde (401.2)	2	OK (402.2)
2-methylpropionaldehyde (387.2)	2	OK (388.2)
2-methyvaleraldehyde (415.2)	2	OK (416.2)
trimethylacetaldehyde (401.2)	2	OK (402.2)
quinoline-4-carboxaldehyde (472.3)	3	OK (402.2)
thiophene-2-carboxaldehyde (427.2)	3	OK (428.2)

13. EXAMPLE: NON-SEQUENTIAL CODING OF A LIBRARY

     We have tested nonsequential coding using both a model test compound and by construction of a nonpeptidic library.

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13.1   INSTRUMENTS, MATERIALS AND PROCEDURES

Instrumentation: Both analytical and preparative
HPLC were carried out on a modular Hitachi system
using Vydac (0.46 x 250mm, 5μm, flow 1ml/min) and
5   Vydac (10 x 250mm, 10μm, flow 3ml/min) C-18 columns,
respectively.

Materials: Unless indicated otherwise,
commercial-grade solvents were used without further
purification.   TentaGel (TG) resin (0.21mmol/g) were
10   received from Rapp-Polymere (Tübingen).   Protected
amino acids were obtained from Bachem (Torrance,
California), Advanced ChemTech (Louisville, Kentucky),
or Propeptide (Vert-le-Petit, France).

More than 100 coding subunits were synthesized on
15   solid phase support. Lysine, ornithine,
diaminobutyric and diaminopropionic acids were used as
base building blocks. Fmoc-Lys(Boc), Fmoc-Orn(Boc),
Fmoc-Dab(Boc) and Boc-Dap(Fmoc) were coupled first to
amino TentaGel (0.21 mmol/g) by the usual DIC/HOBt
20   procedure. After removing the side-chain protection,
modifying carboxylic acids were coupled to the
unprotected side-chains using the DIC/HOBt procedure,
symmetrical anhydrides or acyl chlorides. All new
coding amino acids synthesized were completely
25   deprotected and submitted to Edman degradation in an
Applied Biosystems ABI 477A Protein Sequencer.
Retention times of novel PTH-AA were determined using
an Applied Biosystems ABI 120A Analyzer with a PTH-222
30   Brownlee column (PTH C-18 5 micron, 220 x 2.1 mm).
HPLC buffers: A - 0.01 M NaOAc, B - acetonitrile;
gradient: 0.0-0.4 min - 8% B, 0.4-38.0 min - 8-60% B,
38.0-40.0 min - 60-90% B; flow rate 230 ul/ min.
Peaks were detected at 269 nm. Table 14 lists the
35   individual coding subunits and indicates the numbering
system by which they are referred herein.

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13.2   SYNTHESIS OF MODEL SEQUENCES

The test compound sequences prepared were
Tyr-Gly-Ala-Phe and Phe-Gly-Ala-Phe, coded by doublets
of amino acids (see Scheme XIC), and Tyr-Gly-Gly-Phe-
5   Leu and Phe(Cl)-Gly-Gly-Phe-Leu (encoded by coding by
Scheme XIB). These sequences were selected because
Tyr-Gly-Ala-Phe can be detected using an anti-β-
endorphin antibody as the acceptor molecule and Phe-
Gly-Ala-Phe is a negative control. Moreover, the
10 fidelity and coding could be confirmed by directly
sequencing the test peptides.

13.2.1 MATERIALS AND METHODS

The purpose in synthesizing the model peptides
15 was not to demonstrate a method of library synthesis,
but rather to demonstrate the fidelity of coding.
Therefore, the synthesis was not performed in
successive steps of coupling test AA subunit followed
by a pair of coding subunits, but rather using the
20 more convenient scheme described below. Polymer
carrier (TentaGel, 100mg, 0.21 mmol/g, 90 um average
particle size) onto which the sequence Boc-Ala-Gly-
Val-Phe-bAla-Gly-bAla-Gly was previously synthesized
and which had undergone chymotrypsin treatment to
25 differentiate the surface from the interior by
cleavage of Phe (7mg chymotrypsin in 30 ml of 0.1 M
Tris buffer pH 7.6 in 0.1 M $CaCl_2$, 14 hours at 37°C)
was swollen in dimethylformamide (DMF) (swollen volume
30 0.5 ml), divided into two polypropylene syringes
equipped with polypropylene fritted discs (Krchnak &
Vagner, 1990, J. Pept. Res. 3:182-193), Fmoc-Phe (3
equivalents with respect to all theoretically
available amino groups) was coupled by
35 diisopropylcarbodiimide (DIC) (3 equivalents) in the
presence of N-hydroxybenzotriazole (HOBT) (3

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equivalents) in DMF. After the disappearance of blue
coloration (Krchnák et al., 1988 Coll. Czech. Chem.
Commun. 53:2542), the resin was washed (5x DMF) and
Fmoc group was removed by the treatment of 50%

5   piperidine in DMF (10 min). After washing by DMF (5x)
and 2% HOBT in DMF (1x), the next amino acid was
coupled. In this way alanine, glycine and tyrosine
were attached to resin in the first syringe, and
alanine, glycine and phenylalanine were attached to

10 resin in the second syringe. The Fmoc group was
removed as above and after washing with DMF the resin
was treated with the solution (0.4 M) of 2-
chlorobenzyloxycarbonylsuccinimide in DMF overnight
(after 3h one equivalent of diisopropylethylamine was

15 added). The Boc group was cleaved with 50%
trifluoroacetic acid (TFA) in DCM (1 plus 20 min).
After washing with DCM (3x) and DMF (4x), the resin
was neutralized by washing with 10%
diisopropylethylamine in DMF, washed by DMF (3x) and

20 Fmoc-Lys(Dde) (3 eq) was coupled by the action of DIC
and HOBT (3 eq each) in DMF (2 h). The resin was
washed with DMF (5x) and the Fmoc group was cleaved by
50% piperidine in DMF (20 min), and resin was washed
by DMF (3x). The mixture of amino acids coding for

25 phenylalanine in position four of the test compound
structure (Boc-Sar and Boc-Asp(OBzl) in a molar ratio
of 1:1 (reflecting their relatively equivalent
coupling reactivity which had previously been

30 determined experimentally) was coupled using DIC and
HOBT (3eq each). The reaction was monitored by
bromophenol blue method. The resin was washed by DMF
(5x) and the Dde group was deprotected by 2% hydrazine
hydrate in DMF (10 min). The resin was washed by DMF

35 (5x), 2% HOBT in DMF, and Fmoc-Lys(Dde) was coupled,
and Fmoc group removed. The procedure of coupling

mixtures of Boc amino acids and Fmoc-Lys(Dde) was
repeated two times (coupling the coding subunit
mixtures of Ile and Val (2:1) and Lys(ClZ) and
Glu(OBzl) (1.5:1)) and after deprotection of the Dde
5   group, Boc-Lys(Fmoc) was coupled to the resin in both
syringes. The Fmoc group was deprotected as above and
after washing by DMF (5x) the mixture of butyric and
propionic anhydrides (1:1 in the presence of one
equivalent of triethylamine) were used for acylation
10   of the free amino group in the first syringe and
mixture of 4-phenylbutyric acid and 3-phenylpropionic
acid was coupled to the resin in the second syringe by
the action of DIC and HOBT. The resin was then
deprotected by application of the mixture of TFA
15   (82.5%), p-cresole (5%), thioanisole (5%), water (5%),
and ethanedithiol (2.5%)(mixture K, (King, D. et al.,
1990, Int. J. Pep. Prot. Res. 36:255-266) for 2 hours
and washed by DMF (4x) and 0.1%HCl in water (5x).

20   13.2.2   MODEL COMPOUNDS YGAF AND FGAF
                    CONSTRUCTED WITH CODING
          The two model peptides were synthesized on the
     surface of a "shaved" polyoxyethylene grafted
     polystyrene solid phase support (TentaGel). Coding
25   molecules were synthesized in the interior of the
     TentaGel support and, thus, could not bind to the
     anti-β-endorphin antibody. Differentially seletive
     binding of the positive control test compound was
30   validated using each test compound/bead construct
     alone, or as a mixture of both sequences, or after
     addition of a small number of specific beads to a
     library of compounds.
          A non-sequential code was constructed on a
35   polylysine backbone.

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        The code was as follows:

    Step Subunit   Coding moiety
    4   Phe   butyryl-lysine, proprionyl-lysine
    4   Tyr ϕ-butyryl-lysine, ϕ-propionyl-lysine
    3   Gly   Glu,Lys
 5 2   Ala   Val, Ile
    1   Phe   Asp, Sar

        The positive supports were selected after
10 exposure to the antiβ-endorphin antibody. One cycle
    of Edman degradation identified the positive beads as
    YGAF because of the release of the ϕ-butyryl-lysine,
    ϕ-propionyl-lysine derivatives. Since the test
    compound sequence was present only on the surface of
    the bead, and this amounted to approximately 2% of the
    total amount of molecules attached to the bead, the
15 signal of tyrosine detected in the first cycle (in
    which all the coding amino acid subunits were also
    cleaved) was extremely small. To confirm that we had
    actually detected the proper beads, the sequencing was
    repeated with 30 pooled beads to amplify the signal.
20 In this case four cycles of sequencing directly
    revealed the YGAF test compound sequence.

        **13.3 SYNTHESIS OF THE DIAMINOBENZOIC ACID
           BASED LIBRARY WITH DIGITAL CODING**

25   A library based on the diaminobenzoic acid was
    synthesized and encoded with a non-sequential code.
    The synthetic scheme of this library is given in the
    Scheme below. A list of the amino acids used in the
30 first coupling step and the acids used in coupling
    steps, two and three, to the amino groups of the
    diaminobenzoic acid scaffold are given in Tables 12
    and 13, respectively. Each table also gives the
    coding moieties corresponding to each species subunit
35 of the test compound. The coding moieties in Table 13

are indicated by two digit numbers, e.g. 3/1, the
chemical meanings of which are defined in Table 14.

Sequencing of several randomly chosen beads from
this library confirm the possibility of one step
5 decoding by Edman degradation.

13.3.1 <u>MATERIALS AND METHODS</u>

Synthesis was performed on TentaGel resin (90
μm, 0.2 mmol/g). Fmoc deprotection: 50% piperidine in
10 DMF, 10 min, wash with DMF 6 times, collect all
washes, measure absorbance at 302 nm, calculate Fmoc
release.

Alloc deprotection: Wash 3X DMF (2 min each).
Add mixture of DMF/AcOH/NMM (5ml,1ml,0.5ml) and bubble
15 under Argon for 15 minutes. Add 150 mg
tetrakis(triphenylphospine)palladium and bubble Argon
for 3 hours. Wash 3X DMF. Wash 5X DCM.

Dde deprotection: Washed peptide resin was
treated with 3% solution of hydrazine in DMF for 5 min
20 and 30 min and followed by DMF wash.

Ddz deprotection: Peptide-resin was washed with
DMF then with DCM and pretreated with 3% TFA in DCM
for 5 min, twice. Third treatment with 3% TFA/DCM is
done for 30 min. Thorough wash with DCM is followed
25 by neutralization by 5% DIEA in DCM and then DCM and
DMF wash.

Npys deprotection: Washed peptide resin was
treated with 0.3 M-HCl in dioxane for 5 min. and then
30 30 min. Deprotected peptide-resin was washed with
dioxane, DCM, neutralized with 5% DIEA in DCM, and
washed by DCM and DMF.

Coupling of amino acids: activate 3 molar excess
of protected amino acid by BOP (molar ratio 1:1) in
35 DMF. Check completeness of each condensation reaction

(1.5 - 40 hrs) by ninhydrin test, or by chloranil test
in the cases of coupling to secondary amino groups.

        Side-chain deprotection: Wash with DCM 3 times,
and deprotect using mixture K (82.5% TFA, 5% p-cresol,
5   5% thioanisole, 5% water, 2.5% ethanedithiol) for 5 +
120 min.

        Final washes: neat TFA (3x), DCM (5x), DMF (10x),
DMF/0.1% HCl (5x), 0.1% HCl (3x), and 0.01% HCl (4x).

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13.3.2 SYNTHESIS OF THE LIBRARY

Synthesis: TentaGel S NH2 (10g) was preswollen in DMF and washed 5 times with DMF and subjected to solid phase synthesis using the following protocol which is schematically represented:

Step	Reagent	
1	5% DIEA/DMF	2x5 min
2	DMF wash	10x2 min
3	Fmoc-Lys(Dde)/BOP	until Ninhydrin test is negative
4	50% piperidine/DMF	10 min
5	Fmoc-Lys(Alloc)/BOP	until Ninhydrin test is negative
6	repeat step 4	
7	Boc-Gly/BOP	until Ninhydrin test is negative
8	Pd catalyst	
9	repeat step 7	
10	Fmoc-Gly/BOP	until Ninhydrin test is negative
11	repeat step 4	
12	Fmoc-β-Ala/BOP	until Ninhydrin test is negative
13	repeat step 4	
14	repeat step 10	
15	repeat step 4	
16	repeat step 12	
17	TFA/DCM	
18	randomization and code 1 (table 3) amino acids/BOP	
19	repeat step 4	
20	Ddz-Gly/BOP	
21	3% hydrazine/DMF	
22	Fmoc-Lys(Dde)/BOP	until Ninhydrin test is negative
23	Allyl chloroformate	until Ninhydrin test is negative
24	3% TFA/DCM	
25	Fmoc-amino acids R1/BOP (table 3)	

26	repeat step 4	
27	scaffold I /TBTU	until Ninhydrin test is negative
28	Pd catalyst	
29	randomization + code 2 (table 4)/BOP	
30	HCl/dioxane	
31	47 acids R 2 (table 4)/sym. anhydrides	
32	repeat step 4	
33	randomization + code 3	
34	50 acids R 3 (table 4)/sym. anhydrides	
35	repeat step 21	
36	code 3 (table 4)/BOP	
37	mixture K	
38	final wash	

Protected library was stored in DMA/0.3% HOBt, deprotected library in 0.01% aqueous HCl.

Scheme 5
Scheme of the synthesis of Diaminobenzoic acid (DABA) library with digital code

Fmoc-Lys(Dde)-Tg

| -Fmoc
↓ +Fmoc-Lys(Alloc)

Fmoc-Lys-Lys-TG
      | |
    Alloc   Dde

| -Fmoc
↓ +Fmoc-Lys(Alloc)

Boc-Gly-Lys-Lys-TG
        | |
      Alloc   Dde

| -Alloc (one batch)
↓ Fmoc-β-ala-gly-β-ala-gly

Boc-Gly-Lys-Lys-TG
                        | |
Fmoc-β-ala-gly-β-ala-gly   Dde

| -Boc
↓ Randomization
  +Boc-CODE1 (1st set of 9 code aminoacids)

Boc-CODE1-Gly-Lys-Lys-TG
                          | |
Fmoc-β-ala-gly-β-ala-gly   Dde

| -Fmoc
| +Ddz-gly
| -Dde
| +Fmoc-K(dde)
| -Fmoc
↓ +Alloc

Boc-CODE1-Gly-Lys · · · · · · · · · · · Lys-TG
              | |
          Ddz-gbgbg   Alloc-Lys
                                         |
                                        Dde

| -Ddz
↓ +Fmoc-AA (aminoacids + unusual AA) (R1)

```
              Boc-CODE1-Gly-Lys············· Lys-TG
                           |                    |
              Fmoc-R1-gbgbg                  Alloc-Lys
                                                |
   5                                           Dde

                           |    -Fmoc
                           ↓    +DABA (Fmoc,Npys)


              Boc-CODE1-Gly-Lys················· Lys-TG
                           |                      |
              Fmoc-DABA-R1-gbgbg              Alloc-Lys
  10                |    |                         |
                  Npys (Boc)                      Dde


                           |    -Alloc
                           |    Randomization
                           |    +Boc-CODE2
                           |    -Npys
  15                       ↓    +R2 (47 acids)


              Boc-CODE1-Gly-Lys·············· Lys-TG
                           |                    |
              Fmoc-DABA-R1-gbgbg            Alloc-Lys
                    |    |                       |
                   R2  (Boc)                    Dde
  20                |
                  (Boc)

                           |    -Fmoc
                           |    Randomization
                           |    +R3 (50 acids)
                           |    Dde
                           ↓    +Boc-CODE3
  25
              Boc-CODE1-Gly-Lys·············· Lys-TG
                           |                    |
    (Boc)-R3-DABA-R1-gbgbg              Boc-CODE-Lys
                    |    |                       |
                   R2  (Boc)                 Boc-CODE3
  30                |
                  (Boc)


                         Final deprotection


              CODE1-Gly-Lys················· Lys-TG
                           |                   |
  35      R3-DABA-R1-gbgbg               CODE2-Lys
                    |                          |
                   R2                        CODE3
```

Screening of the Bar Coded Library

The library was screened according to a published
procedure (Lam & Lebl, 1992, Immunomethods 1:11-15).
5 The library beads were first mixed with
incrementally increasing double-distilled water.
After extensive washing with PBS (137 mM NcCl, 2.7
mM KCl, 4.3 Na_2HPO_4, 1.4mM KH_2PO_4, pH 7.2) with 0.1%
Tween-20, the beads were incubated in 0.05% gelatin
10 (w/v) to block any nonspecific binding. The beads
were then incubated with 60pM biotinylated anti-β-
endorphin (clone 3-E 7, Boehringer Mannheim) in 2 x
PBS/Tween/gelatin overnight. After thorough
washing, streptavidin-alkaline phosphatase was
15 added. One hour later, the beads were washed, and
the standard substrate for alkaline phosphatase, 5-
bromo-4-chloro-3-indolyl phosphate, was added. The
beads together with the substrate, were then
transferred to petri dishes for color development.
20 After 30 minutes to 1 hour, the positively colored
beads were collected using a micropipette, washed
with 6M guanidine hydrochloride, pH 1.0, and
subjected to sequencing of the bar code.

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Table 12

Amino Acid Doublets Used for Encoding Block R1

R1= 29 Amino Acids

Amino Acid	Code Elements
Gly	Ala Phe
Ala	Ala Ile
Dap	Aib Val
Pro	Aib Phe
Val	Aib Ile
Pipecolic acid	Val Phe
Leu	Val Ile
Asn	Phe Ile
Asp	Asn Gln
Orn	Asn Gly
Gln	Asn Ala
Glu	Asn Aib
2-Pyridyl-Ala	Asn Val
Chg	Asn Phe
Phe	Asn Ile
Cha	Gln Gly
Arg	Gln Ala
Citruline	Gln Aib
Tetrahydroisoquinoline carboxylic acid	Gln Val
homoPhe	Gln Phe
N-Me-Gly	Gln Ile
Phe(p-F)	Gly Ala
Phe(p-Cl)	Gly Aib
Trp	Gly Val
Phe(p-NO2)	Gly Phe
Ala(1-Naph)	Gly Ile
3,4-dichloro-Phe	Ala Aib
Lys(TFA)	Ala Val
Phe(p-Bz)	Ala Phe

Aib =   aminoisobutyric acid

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Table 13

Acids used for randomizations of positions R2 and R3
(see scheme 1)

R2 = 47 Acids (this set less A16, A28 & A47)

R3 = 50 Acids

	Subunits of Test Compound	Coding Amino Acids	
A01	Acetic acid	1/2	1/3
A02	Propionic acid	1/2	3/1
A03	Hexanoic acid	1/2	3/3
A04	Isobutyric acid	1/2	3/4
A05	Trimethylacetic acid	1/2	NVal
A06	Cyclopentanecarboxylic acid	1/2	Leu
A07	Cyclohexanecarboxylic acid	1/2	Nle
A08	Cyclohexylacetic acid	1/2	PheCl
A9	1-Adamantaneacetic acid	1/2	15/4
A10	Glycine	1/2	2'Nal
A11	β-Alanine	1/3	3/1
A12	ϵ aminocaproic acid	1/3	3/3
A13	γ-Guanidinobutyric acid	1/3	3/4
A14	Serine	1/3	NVal
A15	Threonine	1/3	Leu
A16	Cys(SMe)	1/3	Nle
A17	Succinic acid	1/3	PheCl
A18	Glutaric acid	1/3	15/4
A19	cis-1,2-cyclohexanedicarboxylic acid	1/3	2'Nal
A20	Succinamic acid	3/1	3/3

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	Subunits of Test Compound	Coding Amino Acids	
A21	Benzoic acid	3/1	3/4
A22	1-Naphthylacetic acid	3/1	NVal
A23	Biphenylacetic acid	3/1	Leu
A24	Dipenylacetic acid	3/1	Nle
A25	4-Aminobenzoic acid	3/1	PheCl
A26	4-Dimethylaminobenzoic acid	3/1	15/4
A27	4-Guanidinobenzoic acid	3/1	2'Nal
A28	3,4-dichlorophenylalanine	3/3	3/4
A29	4-Nitrophenylacetic acid	3/3	Nval
A30	4,5-dimethoxy-2-nitrobenzoic acid	3/3	Leu
A31	4-Chlorobenzoic acid	3/3	Nle
A32	α,α,α Trifluoro-p-toluic acid	3/3	PheCl
A33	4-Hydroxybenzoic acid	3/3	15/4
A34	4-Hydroxyphenyl acetic acid	3/3	2'Nal
A35	3-(3,4,5-trimethoxyphenyl)proionic acid	3/4	Nval
A36	4-(3-methyl-5-oxo-2-pyrazolin-1-yl) benzoic acid	3/4	Leu
A37	Proline	3/4	Nle
A38	3-carboxyl-1,4-dimethyl-2-pyrroleacetic acid	3/4	PheCl
A39	2-Methyl-4-nitro-1-imidazolepropionic acid	3/4	15/4
A40	2-Amino-1-imidazoleacetic acid	3/4	2'Nal

	Subunits of Test Compound	Coding Amino Acids	
A41	3-Amino-1,2,4-triazole-5-carboxylic acid	NVal	Leu
A42	4-Imidizoleacetic acid	NVal	Nle
A43	Isonicotinic acid	NVal	PheCl
A44	2,3-Pyridinedicarboxylic acid	NVal	15/4
A45	2-Pyrazinecarboxylic acid	NVal	2'Nal
A46	2,3-Pyrazinedicarboxylic acid	Leu	Nle
A47	Pipecolic acid	Leu	PheCl
A48	1-Methylindole-2-carboxylic acid	Leu	15/4
A49	2-Methyl-3-indoleacetic acid	Leu	2'Nal
A50	Indole-4-carboxylic acid	Nle	PheCl

Table 14

Amino Acid and Carboxylic Acid Matrix

Species No.	α,β-Diamino propionic Acid(1)	α,γ-Diamino butyric Acid(2)	ornithine (3)	lysine (4)	Derivative
1	*	*	*	*	acetyl
2	*	*	*	*	propionyl
3	*	*	*	*	butyryl
4	*	*	*	*	valeryl
5	*	*	*	*	caproyl
6	*	*	*	*	pivaloyl
7		*	*	*	c-hexanoyl
8		*	*	*	trichloroacetyl
10		*	*	*	phenylacetyl
11		*	*	*	2,2-diphenylacetyl
12		*	*	*	phenylbutyryl
13		*	*	*	1-naphtylacetyl
14		*	*	*	2-naphtylacetyl
15	*	*	*	*	1-adamantylcarbonyl
16		*	*	*	1-adamantylacetyl
17		*	*	*	tosylglycyl
18		*	*	*	dansylglycyl
19	*	*	*	*	benzoyl
20	*	*	*	*	succinamyl
21		*	*	*	succinyl
22		*	*	*	glutaryl
23	*	*	*	*	isobutyryl
24		*	*	*	4-chlorobenzoyl
25		*	*	*	2,2-diphenylpropionyl

Species No.	α,β-Diamino propionic Acid(1)	α,γ-Diamino butyric Acid(2)	ornithine (3)	lysine (4)	Derivative
26		*	*	*	N,N-dimethylglycyl
27	*	*	*	*	heptanoyl
28	*	*	*	*	octanoyl
29	*	*	*	*	3,3-di-ph-propionyl
30		*	*	*	N,N-dimethylaminobutyryl
31	*	*	*	*	3-ph-propionyl
32	*	*	*	*	4-bi-ph-carbonyl
33	*	*	*	*	4-bi-ph-acetyl
34	*	*	*	*	crotonoyl

(* means that the derivative was synthesized and analysed)

Code explanation: e.g. 19/4 means Lys(benzoyl)

14.   EXAMPLE: MOLECULAR SCAFFOLDS

SCHEME XIX provides the chemical structures of 19 compounds that can be used to construct test compounds. The scheme indicates the site of attachment of the subunits of the test compounds by R_s.

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Scheme XIX:

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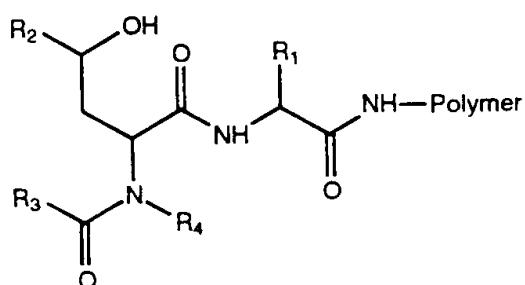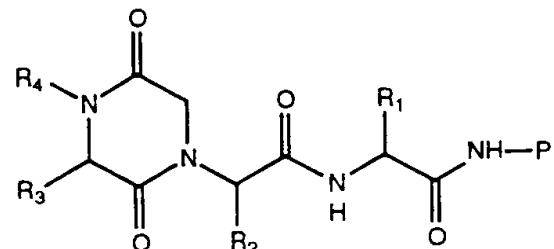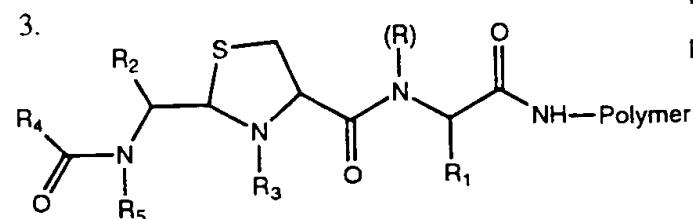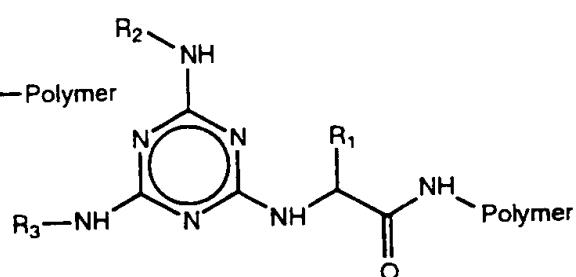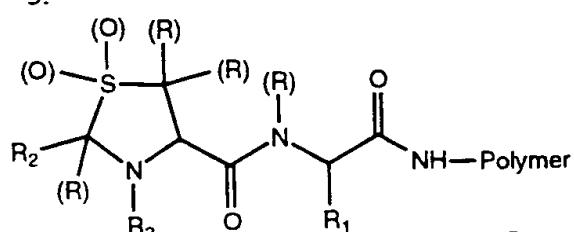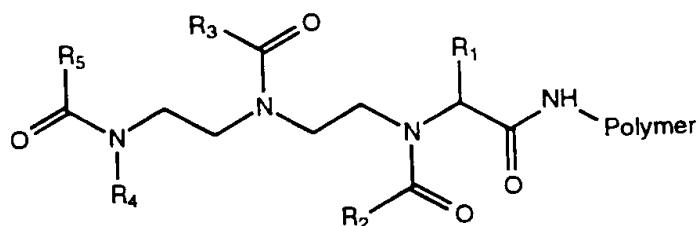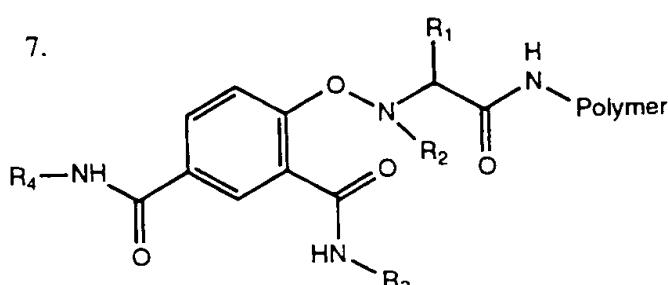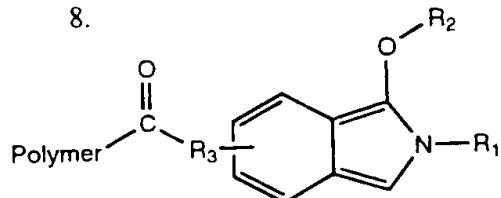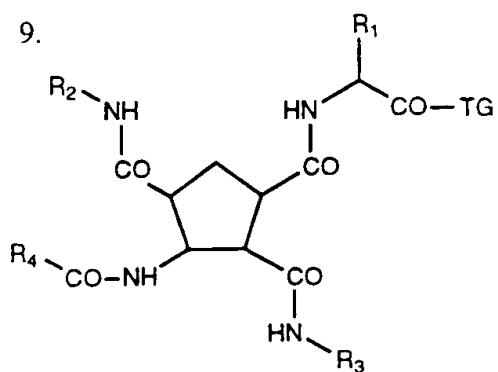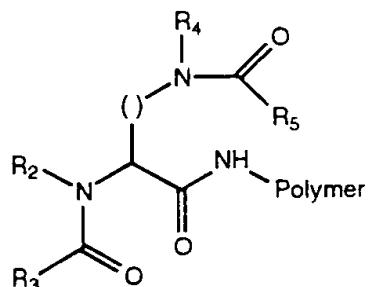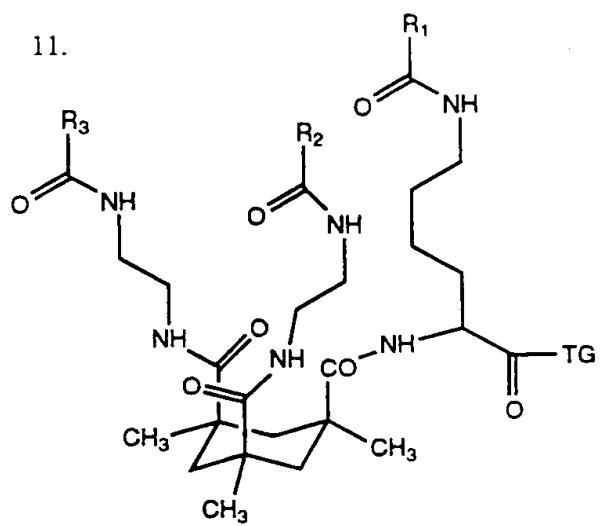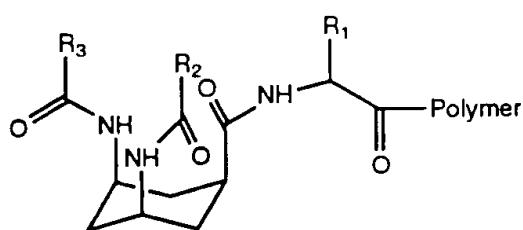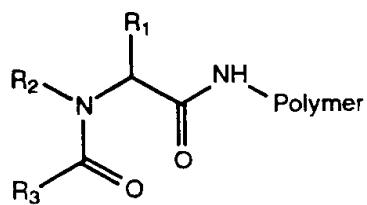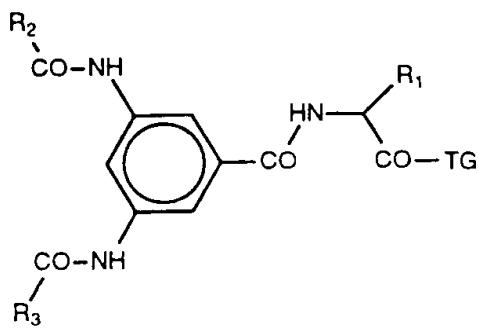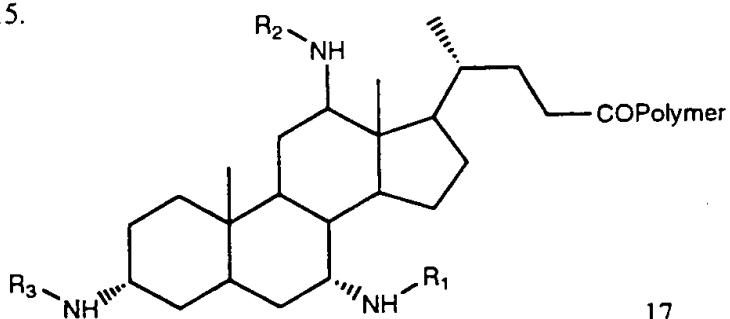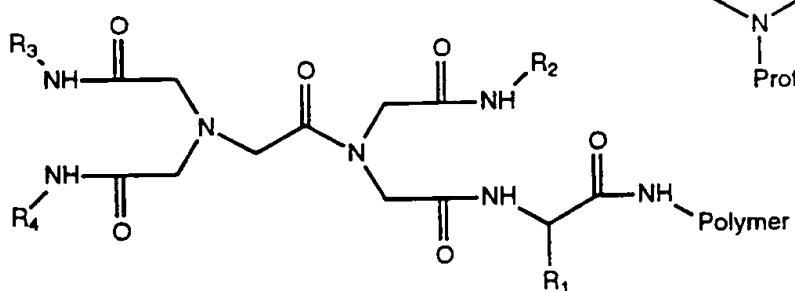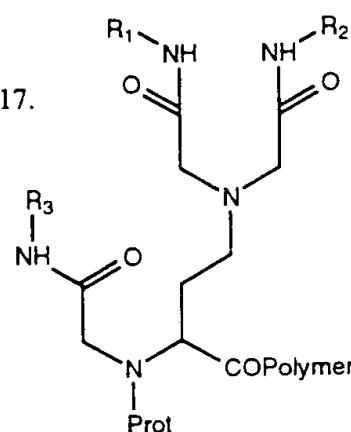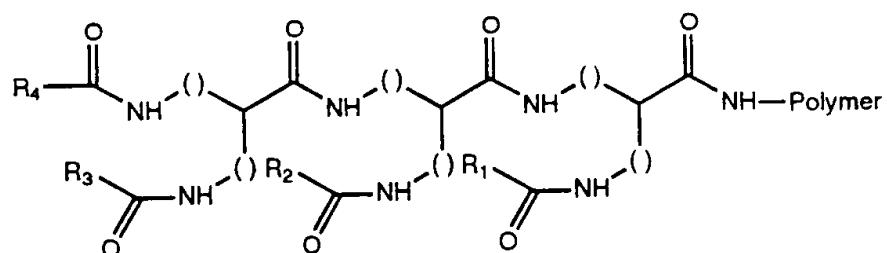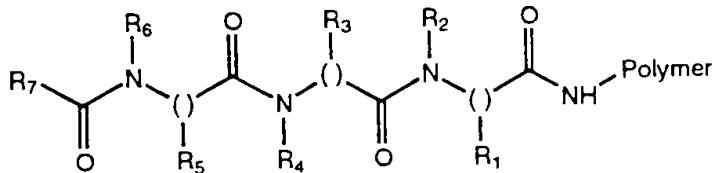
Table 15 below provides guidance concerning the
types of subunits and the chemistry of their
attachment to the molecular scaffold.

Scaffold No.	Subunits	Chemistry of Coupling
1. Amino Acid Aldehyde/ Organo-metal	amino acid homoserine aldehyde alkyl- or arylmetal	CO-NH coupling C-C bond formation
2. Diketo- piperazine	amino acids N-alkyl amino acids	CO-NH coupling reductive amination
3. Substituted Thioproline	amino acids cysteine amino acid aldehyde alkyl or aryl acids	CO-NH coupling reductive amination
4. Substituted Triazine	amino acid trichlorotriazine alkyl or aryl amines	CO-NH coupling reductive amination
5. Substituted Thioproline Dioxide	amino acids N-alkyl amino acids cysteine aldehyde, ketone	CO-NH coupling thioaminal formation oxidation C-alkylation
6. Acylated Polyethylene- Diamine	amino acids glycinal alkyl or aryl acids	CO-NH coupling reductive amination
7. Benzenetricarbo xylic Acid	amino acids N-alkyl amino acids 1,2, 4-benzenetri- carboxylic acid	CO-NH coupling
8. 2-S-alkyl (aryl) isoindol	subst. phthalic anhydride alkyl or aryl amines alkyl or aryl mercaptanes	isoindol synthesis
9. Cyclopentane	N-alkyl amino acids prim. or sec. amines cyclopentantricarbox ylic acid	CO-NH coupling
10. Diacyldialkyl Diamino Acid	amino acids aldehydes alkyl or aryl acids	CO-NH coupling reductive amination

Scaffold No.	Subunits	Chemistry of Coupling
11. Extended Kemps Triacid	amino acids Kemp's triacid protected diamines	CO-NH coupling
12. Kemps Triacid	amino acids Kemp's triacid alkyl or aryl acids	CO-NH coupling
13. Akyl Acyl Amino Acid	amino acids aldehydes alkyl or aryl acids	CO-NH coupling
14. Diaminobenzoic Acid	amino acids 3, 5-diaminobenzoic acid alkyl or aryl acids	CO-NH coupling
15. Steroid	steroid skeleton aldehydes	reductive amination CO-NH coupling
16. Bis-Iminodiacetic Acid	glycine t-butylbromoacetate alkyl or aryl amines	
17. N-alkylated Iminodiacetic Acid	diaminobutanoic acid t-butylbromoacetate alkyl or aryl amines	CO-NH coupling N-alkylation
18. α,β,γ Peptidomimetic	diaminoacids alkyl or aryl acids	CO-NH coupling
19. N-Substituted Glycine Peptidomimetic	amino acids aldehydes	CO-NH coupling reductive amination

WHAT IS CLAIMED IS:

      1.   A library for identifying and analyzing a ligand of an acceptor of interest comprising: a multiplicity of separate solid phase supports; to each of which are attached:

      a)   a species of test compound, said test compound comprised of a sequence of subunits; and

      b)   a species of coding molecule, wherein said coding molecules are topologically segregated from said test compounds and said species of coding molecules are attached by a multiplicity of successive, separate chemical reactions; and, further, wherein the sequence of the subunits of the test compound attached to a particular solid phase support is encoded by said species of coding molecule attached to the particular solid phase support.

      2.   The library of claim 1 in which the test compound is non-sequencable.

      3.   The library of claim 2 in which the test compound is a polymer.

      4.   The library of claim 3 in which the test compound is a polymer selected from the group consisting of polyamide, polyester, polyurea, polyurethane, polycarbonate, polyamine, polyalkane, polyalkene, polyalcohol, polysulfide and polydisulfide.

      5.   The library of claim 2 in which the subunits of the test compound are linked by chemical

bonds selected from the group consisting of amide, ester, urea, urethane, carbonate, amine, alkane, alkene, sulfide, and disulfide bonds.

5   6.   The library of claim 2 in which the test compound is further comprised of a molecular scaffold.

7.   The library of claim 6 in which the
10 molecular scaffold is selected from the group consisting of a steroid structure, a sugar, a heterocyclic structure, and a polyaromatic compound.

8.   The library of claim 6 in which the
15 molecular scaffold is an Amino Acid Aldehyde/Organo-metal, a Diketopiperazine, a Substituted Thioproline, a Substituted Triazine, a Substituted Thioproline Dioxide, an Acylated Polyethylene-Diamine, a Benzenetricarboxylic Acid, a 2-S-alkyl
20 (aryl) isoindol, a Cyclopentane, a Diacyldialkyl Diamino Acid, an Extended Kemps Triacid, a Kemps Triacid, an Akyl Acyl Amino Acid, a Diaminobenzoic Acid, a Steroid, a Bis-Iminodiacetic Acid, an N-alkylated, an Iminodiacetic Acid, an α,β,γ
25 Peptidomimetic, or an N-Substituted Glycine Peptidomimetic.

9.   The library of claim 1 in which the
30 test compound is a polymer.

10.   The library of claim 1 in which the coding molecule is comprised of α-amino acids.

35   11.   The library of claim 10 in which the coding molecule is a linear polypeptide.

12.   The library of claim 10 in which the coding molecule is a branched polypeptide.

13.   The library of claim 2 in which a sequence of subunits of a test compound of a particular support is encoded by a single species of coding molecule attached to said suppport.

14.   The library of claim 2 in which a sequence of subunits of a test compound of a particular support is encoded by a plurality of species of coding molecules attached to said support.

15.   The library of claim 2 in which a sequence of subunits of a test compound of a particular support is encoded by a code comprising an order of the successive reactions that attached each said species of coding molecules.

16.   The library of claim 15 in which the sequence of subunits of the test compound is encoded by a single species of coding molecule.

17.   The library of claim 15 in which the sequence of subunits of the test compound is encoded by a plurality of species of coding molecules.

18.   The library of claim 1 in which the sequence of subunits of the test compound is encoded by a code in which the order of the successive reactions that attached each said species of coding molecules is irrelevant.

19.   The library of claim 18 in which the
sequence of subunits of the test compound is encoded
by a single species of coding molecule.

5   20.   The library of claim 18 in which the
sequence of subunits of the test compound is encoded
by a plurality of species of coding molecules.

21.   The library of claim 18 in which the
10   coding molecule comprises a polymer of amino acids,
said polymeric amino acids having:

    a)   a first amino moiety whereby a peptide
        bond is formed linking said polymeric
        amino acids to each other; and

15   b)   a second amino moiety, whereby one of a
        multiplicity of species of α-amino
        acids are coupled to each of said
        polymeric amino moieties by a peptide
        bond.

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22.   The library of claim 21 in which the
first amino moiety is an Nα amino moiety and the
second amino moiety is selected from the group
consisting of Nβ, Nγ, Nδ and Nϵ.

25

23.   The library of claim 21 in which the α-
amino acids coupled to said second amino moieties
comprise derivatives of α,β-diaminoproprionic acid,
30   α,γ-diaminobutyric acid, ornithine and lysine,
glutamic acid, aspartic acid, cysteine and
penicilamine.

24.   The library of claim 23 in which the
35   derivatives are formed by reaction of a carboxylic
acid with an Nβ, Nγ, Nδ or Nϵ amino to form a
derivative selected from the group consisting of

acetyl, propionyl, butyryl, valeryl, caproyl,
pivaloyl, c-hexyl, trichloroacetyl, phenylacetyl,
2,2-diphenylacetyl, phenylbutyryl, 1-naphtylacetyl,
2-naphtylacetyl, 1-adamantylcarbonyl, 1-
5   adamantylacetyl, tosylglycyl, dansylglycyl, benzoyl,
succinamyl, succinyl, glutaryl, isobutyryl, 4-
chlorobenzoyl, 2,2-diphenylpropionyl, N,N-
dimethylglycyl, heptanoyl, octanoyl, 3,3-di-ph-
propionyl, N,N-dimethylaminobutyryl, 3-ph-propionyl,
10 4-bi-ph-carbonyl, 4-bi-ph-acetyl and crotonyl.

      25.   The library of claim 2 in which a
subunit of a species of test compound is attached by
nucleophilic displacement and is selected from the
15 group consisting of Ethylamine, i-Propylamine,
Butylamine, i-Butylamine, Cyclopentylamine,
Cyclohexylamine, Ethanolamine, 3-Aminopropanol, 1-
Amino-2-propanol, 2-Methoxyethylamine, β-Ala-OtBu,
Ethylendiamine(Boc), 2-(2-Aminoethyl)1-
20 methylpyrrolidine, Benzylamine, Naphthalene-
methylamine, 4-(Trifluoromethyl)-benzylamine, 2-
Amino-1-phenyl-ethanol, Tyramine, 4-Methoxy-
benzylamine, 3,5-Dimethoxy-benzylamine and 4-
(Dimethylamino)-benzylamine.

25

      26.   The library of claim 2 in which a
subunit of a species of test compound is attached by
acylation of a primary amine and is selected from
30 the group consisting of γ-Guanidinobutyric,
Succinamic, 1-Naphtylacetic, Diphenylacetic,
Biphenylacetic, Pentafluorophenylacetic, 4-
Trifluoromethylbenzoic, 4-Hydroxynbenzoic, 4-
Hydroxyphenylacetic, 4-Aminophenylacetic, 3-
35 Nitrophenylacetic, 2-Nitro-4,5,-dimethoxybenzoic, 3-
(3,4,5-trimethoxyphenyl)propionic, 4-
Guanidinobenzoic, 4-Dimethylaminobenzoic, 4-(3-

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Methyl-5-oxo-2-pyrazolin-1-yl)benzoic, 1,4-Dimethyl-
2,3-pyrroledicarboxylic, 2-Methyl-4-nitro-1-
imidazolepropionic, 2-Amino-1-imidazoleacetic, 3-
amino-1,2,4-triazole-5-carboxylic, 4-
5   Imidazoleacetic, 2,3-Pyridinedicarboxylic, 2-
Pyrazinecarboxylic, 2,3-Pyrazinedicarboxylic, 1-
Methylindole-2-carboxylic, 2-Methyl-3-indoleacetic
and Indole-4-carboxylic.

10   27.   The library of claim 2 in which a
subunit of a species of test compound is attached by
reductive alkylation and is selected from the group
consisting of 2-methylbutyraldehyde, 2-
ethylbutyraldehyde, trimethylacetaldehyde, 2-
15   methylvaleraldehyde, cyclohexanecarboxaldehyde,
benzaldehyde, 4-nitrobenzaldehyde, 4-
hydroxybenzaldehyde,
vanillin, 2-thiophenecarboxaldehyde, pyridine-4-
carboxaldehyde, α,α,α-trifluoro-o-tolualdehyde, 4-
20   methoxybenzaldehyde, 1-acetylindole-3-caboxaldehyde,
4-carboxybenzaldehyde, beta-naphtaldehyde, 4-
phenylbenzaldehyde, 3-phenoxybenzaldehyde and 2-
hydroxybenzaldehyde.

25   28.   A method of preparing an encoded
library of a multiplicity of separate solid phase
supports having linkers attached thereto, for
identifying and characterizing a ligand of an
30   acceptor of interest comprising the steps of:

           (a)   coupling, in a plurality of separate
                    reactions of a plurality of aliquots of
                    solid phase supports, a plurality of
35   species of subunits to the linker or to
                    a subunit coupled to the linker, so

that in each reaction only one species
of subunit is coupled;

(b)   attaching a plurality of species of
coding molecules to the plurality of

5   aliquots of the solid phase supports,
in which the species attached to each
aliquot encode the subunit coupled to
the aliquot;

(c)   mixing the aliquots of coupled supports

10   of the separate reactions; and

(d)   performing steps (a) (b) and (c), at
least twice, and as many times as
desired.

15   29.   A method of preparing an encoded
library of a multiplicity of separate solid phase
supports having molecular scaffolds attached
thereto, for identifying and analyzing a ligand of
an acceptor of interest comprising the steps of:

20

(a)   coupling, in a plurality of separate
reactions of a plurality of aliquots of
solid phase supports, a plurality of
species of subunit to the scaffold or

25   to a subunit coupled to the scaffold,
so that in each reaction only one
species of subunit is coupled;

(b)   attaching a plurality of species of

30   coding molecules to the plurality of
aliquots of the solid phase supports,
in which the species attached to each
aliquot encode the subunit coupled to
the aliquot;

35
(c)   mixing the aliquots of coupled supports
of the separate reactions; and

(d)   performing steps (a) (b) and (c), at
           least twice, and as many times as
           desired.

5   30.   A method of screening a library of test
compounds and determining a sequence of subunits of
a species of test compound that binds to an acceptor
molecule of interest comprising the steps of:

10   (a)   introducing an acceptor molecule into a
                library comprising: a multiplicity of
                separate solid phase supports; to each
                of which are attached:
                         i) a species of test compound,
15   said test compound comprised of
                         a sequence of subunits and
                         ii) a species of coding
                         molecule, wherein said coding
                         molecules are topologically
20   segregated from said test
                         compounds and said species of
                         coding molecules are attached by
                         a multiplicity of successive,
                         separate chemical reactions;
25   and, further,
                wherein the sequence of the subunits of
                the test compound attached to a
                particular solid phase support is
30   encoded by said species of coding
                molecule attached to the particular
                solid phase support;
     (b)   identifying a solid phase support that
                binds the acceptor molecule;
35   (c)   isolating an identified solid phase
                supports free of all other supports of
                the library;

(d)   characterizing said species of coding
        molecules attached to the isolated
        solid phase support; and

(e)   decoding the sequence of the subunits
        of the species of test compound
        attached to the isolated solid phase
        support.

31.   The method of claim 30 wherein said
species of coding molecules are characterized by
sequencing the coding molecules.

32.   The method of claim 30 wherein the
coding molecules are comprised of α-amino acids.

33.   The method of claim 32 wherein the
coding molecules are linear polypeptides.

34.   The method of claim 32 where the coding
molecules are branched polypeptides.

35.   The method of claim 34 wherein the
coding molecule comprises a polymer of amino acids,
said polymeric amino acids having:

a)   a first amino moiety whereby a peptide
        bond is formed linking said polymeric
        amino acids to each other; and

b)   a second amino moiety, whereby one of a
        multiplicity of species of α-amino
        acids are coupled to each of said
        polymeric amino moieties by a peptide
        bond.

36.   The method of claim 36 wherein the
first amino moiety is an N_α amino moiety and the

second amino moiety is selected from the group
consisting of N_β, N_γ, N_δ and N_ϵ.

37.   The method of claim 36 wherein the α-
amino acids coupled to said second amino moieties
comprise derivatives of α,β-diaminoproprionic acid,
α,γ-diaminobutyric acid, ornithine and lysine.

38.   The method of claim 37 wherein the
derivatives are formed by reaction of a carboxylic
acid with an N_β, N_γ, N_δ or N_ϵ amino to form a
derivative selected from the group consisting of
acetyl, propionyl, butyryl, valeryl, caproyl,
pivaloyl, c-hexyl, trichloroacetyl, phenylacetyl,
2,2-diphenylacetyl, phenylbutyryl, 1-naphtylacetyl,
2-naphtylacetyl, 1-adamantylcarbonyl, 1-
adamantylacetyl, tosylglycyl, dansylglycyl, benzoyl,
succinamyl, succinyl, glutaryl, isobutyryl, 4-
chlorobenzoyl, 2,2-diphenylpropionyl, N,N-
dimethylglycyl, heptanoyl, octanoyl, 3,3-di-ph-
propionyl, N,N-dimethylaminobutyryl, 3-ph-propionyl,
4-bi-ph-carbonyl, 4-bi-ph-acetyl and crotonyl.

39.   A method of screening a library of
species of test compounds and determining a sequence
of subunits of a species of ligand that binds to an
acceptor molecule of interest comprising the steps
of:

    (a)   providing, in the presence of an
            acceptor molecule of interest, a
            multiplicity of species of test
            compounds, said species of test
            compounds having been released from a
            library comprising: a multiplicity of

separate solid phase supports; to each
of which are attached:

1) a species of test compound,
said test compound comprised of
a sequence of subunits and

2) a species of non-releasible
coding molecules, wherein said
species of coding molecules are
attached by a multiplicity of
successive, separate chemical
reactions; and

in which the sequence of the subunits
of the test compound attached to a
particular solid phase support is
encoded by said species of coding
molecules attached to the particular
solid phase support;

(b)   identifying a solid phase support,
having released therefrom a species of
test compound that binds the acceptor
molecule;

(c)   isolating the identified solid phase
support free of all other supports of
the library;

(d)   characterizing said species of coding
molecules attached to the isolated
solid phase support; and

(e)   decoding the sequence of the species of
subunits of the test compound.

40.   The method of claim 39 wherein said
species of coding molecules are characterized by
sequencing the coding molecules.

41.   The method of claim 40 wherein the
coding molecules are comprised of α-amino acids.

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42.   The method of claim 41 wherein the
coding molecules are linear polypeptides.

43.   The method of claim 41 wherein the
5 coding molecules are branched polypeptides.

44.   The method of claim 43 wherein the
coding molecule comprises a polypeptide of α-amino
acids having a second amino moiety, wherein one of a
10 multiplicity of species of α-amino acids are coupled
to each of said second amino moieties by a peptide
bond.

45.   The method of claim 44 wherein the α-
15 amino acids coupled to said second amino moieties
comprise derivatives of α,β-diaminoproprionic acid,
α,γ-diaminobutyric acid, ornithine and lysine.

46.   The method of claim 45 wherein the
20 derivatives are formed by reaction of a carboxylic
acid with an N_β, N_γ, N_δ or N_ϵ amino to form a
derivative selected from the group consisting of
acetyl, propionyl, butyryl, valeryl, caproyl,
pivaloyl, c-hexyl, trichloroacetyl, phenylacetyl,
25 2,2-diphenylacetyl, phenylbutyryl, 1-naphtylacetyl,
2-naphtylacetyl, 1-adamantylcarbonyl, 1-
adamantylacetyl, tosylglycyl, dansylglycyl, benzoyl,
succinamyl, succinyl, glutaryl, isobutyryl, 4-
30 chlorobenzoyl, 2,2-diphenylpropionyl, N,N-
dimethylglycyl, heptanoyl, octanoyl, 3,3-di-ph-
propionyl, N,N-dimethylaminobutyryl, 3-ph-propionyl,
4-bi-ph-carbonyl, 4-bi-ph-acetyl and crotonyl.

35   47.   A library for identifying and analyzing
a ligand of an acceptor of interest comprising a
multiplicity of solid phase supports to each of

which is attached a non-sequencable test compound
comprised of a sequence of subunits.

5   48.   The library of claim 47 in which a
subunit of a species of test compound is attached by
nucleophilic displacement and is selected from the
group consisting of Ethylamine, i-Propylamine,
Butylamine, i-Butylamine, Cyclopentylamine,
Cyclohexylamine, Ethanolamine, 3-Aminopropanol, 1-
10   Amino-2-propanol, 2-Methoxyethylamine, β-Ala-OtBu,
Ethylendiamine(Boc), 2-(2-Aminoethyl)1-
methylpyrrolidine, Benzylamine, Naphthalene-
methylamine, 4-(Trifluoromethyl)-benzylamine, 2-
Amino-1-phenyl-ethanol, Tyramine, 4-Methoxy-
15   benzylamine, 3,5-Dimethoxy-benzylamine and 4-
(Dimethylamino)-benzylamine.

       49.   The library of claim 47 in which a
subunit of a species of test compound is attached by
20   acylation of a primary amine and is selected from
the group consisting of γ-Guanidinobutyric,
Succinamic, 1-Naphtylacetic, Diphenylacetic,
Biphenylacetic, Pentafluorophenylacetic, 4-
Trifluoromethylbenzoic, 4-Hydroxynbenzoic, 4-
25   Hydroxyphenylacetic, 4-Aminophenylacetic, 3-
Nitrophenylacetic, 2-Nitro-4,5,-dimethoxybenzoic, 3-
(3,4,5-trimethoxyphenyl)propionic, 4-
Guanidinobenzoic, 4-Dimethylaminobenzoic, 4-(3-
30   Methyl-5-oxo-2-pyrazolin-1-yl)benzoic, 1,4-Dimethyl-
2,3-pyrroledicarboxylic, 2-Methyl-4-nitro-1-
imidazolepropionic, 2-Amino-1-imidazoleacetic, 3-
amino-1,2,4-triazole-5-carboxylic, 4-
Imidazoleacetic, 2,3-Pyridinedicarboxylic, 2-
35   Pyrazinecarboxylic, 2,3-Pyrazinedicarboxylic, 1-
Methylindole-2-carboxylic, 2-Methyl-3-indoleacetic
and Indole-4-carboxylic.

50.   The library of claim 47 in which a
subunit of a species of test compound is attached by
reductive alkylation and is selected from the group
consisting of 2-methylbutyraldehyde, 2-
5 ethylbutyraldehyde, trimethylacetaldehyde, 2-
methylvaleraldehyde, cyclohexanecarboxaldehyde,
benzaldehyde, 4-nitrobenzaldehyde, 4-
hydroxybenzaldehyde,
vanillin, 2-thiophenecarboxaldehyde, pyridine-4-
10 carboxaldehyde, α,α,α-trifluoro-o-tolualdehyde, 4-
methoxybenzaldehyde, 1-acetylindole-3-caboxaldehyde,
4-carboxybenzaldehyde, beta-naphtaldehyde, 4-
phenylbenzaldehyde, 3-phenoxybenzaldehyde and 2-
hydroxybenzaldehyde.

15

51.   The library of claim 47 in which the
test compound further comprises a molecular
scaffold.

20   52.   The library of claim 51 in which the
molecular scaffold is an Amino Acid Aldehyde/Organo-
metal, a Diketopiperazine, a Substituted
Thioproline, a Substituted Triazine, a Substituted
Thioproline Dioxide, an Acylated Polyethylene-
25 Diamine, a Benzenetricarboxylic Acid, a 2-S-alkyl
(aryl) isoindol, a Cyclopentane, a Diacyldialkyl
Diamino Acid, an Extended Kemps Triacid, a Kemps
Triacid, an Akyl Acyl Amino Acid, a Diaminobenzoic
30 Acid, a Steroid, a Bis-Iminodiacetic Acid, an N-
alkylated, an Iminodiacetic Acid, an α,β,γ
Peptidomimetic, or an N-Substituted Glycine
Peptidomimetic.

35   53.   A method of topologically segregating a
first molecular species and a second molecular
species, attached to a solid phase support, said

support having a linker attached thereto, said
linker having an enzymatically susceptible bond and
a first protective group, comprising the steps of:

       a.   exposing the support to the enzyme
5   whereby less than all of the first
                protective group is removed;

       b.   coupling the enzymatically exposed
                supports with a subunit having a second
                protective group, said second group
10   being orthogonal to said first group;
                and thereafter

synthesizing on the support the first and the second
molecular species.

15   54.   The method of claim 53 in which the
linker is a peptide and the enzyme is an
endopeptidase.